A NOVEL MODE OF BACTERIAL RESPIRATION: IRON SOLUBILIZATION PRIOR TO ELECTRON TRANSFER

A Dissertation
Presented to
The Academic Faculty

by

Christine Michelle Fennessey

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Applied Biology

Georgia Institute of Technology
December 2010

A NOVEL MODE OF BACTERIAL RESPIRATION: IRON SOLUBILIZATION PRIOR TO ELECTRON TRANSFER

Approved by:

Dr. Thomas J. DiChristina, Advisor
School of Biology
Georgia Institute of Technology

Dr. Roger Wartell
School of Biology
Georgia Institute of Technology

Dr. Jim Spain
School of Biology
Georgia Institute of Technology

Dr. Martial Taillefert
School of Earth and Atmospheric Sciences
Georgia Institute of Technology

Dr. Andrew L. Neal
Centre for Soils and Ecosystem Function
Rothamstead Research, UK

Date Approved: September 1, 2010

“When I consider… the work of your fingers… what is man that you are mindful of him, the son of man that you care for him?” Psalm 8:3-4

For Shelley, my best friend and the beloved of my heart.

ACKNOWLEDGEMENTS

Many thanks to my advisor, Dr. Tom DiChristina. Thank you for taking a chance on a random student in your class. You showed immense understanding and patience and pushed me to think outside the box and challenge dogma. You always made me feel like my ideas were valid and worth pursuing. Thank you for giving me the opportunity to strive toward my full potential as a scientist.

Thank you to the brilliant scientists of my committee: Martial Taillefert, Andy Neal, Jim Spain, and Roger Wartell. Thank you for your time, patience and encouragement in this endeavor.

To my parents, thank you for all your encouragement, for your immense pride in me even when I didn't think I deserved it, for your years of prayers, and for believing in me when I didn't believe in myself. God has blessed me immeasurably by giving me such wonderfully supportive parents. To my beloved little sister, you are the apple of my eye. Thank you for your hugs, our DD dates, and your joyful, generous heart. You mean more to me than I can express. To my brothers, thank you for being such strong, solid men on whom I can always rely. Your brilliance inspires me to work harder to try and reach your level. To the original Dr. Fennessey, thank you for recognizing my potential to be more than sweet and pretty. To my Granddad, thank you so much for your generosity and pride in me. Much love to the rest of my family.

Thanks to my labmates past and present: Justin Burns, Morris Jones, Jason Dale, David Bates, Amanda Payne, Omar Elizondo, Nadia Szeinbaum, Seng Kew Wee and Mia

Mattioli: you are all intelligent and driven, and I have no doubt you'll accomplish great things. It's been an honor to work beside you. Also many thanks to Paul Aphivantrakul, Ewelina Kieley, and Jordon Beckler for lab assistance.

To all the other people in my life who have added joy to the last 5 years of life: Kristen Kitagawa, Ashley Bryant, Fernie Goh, Jacy Gresko, Glenn Fisher, Alex Teodorescu, Ben Hamilton, Chen Cheng, Michelle Neveux, Marisa Davison, Paul Wissmann, my small group and my church, thank you for being wonderful to me!

Funding was provided by the National Science Foundation and the Department of Energy.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF ABBREVIATIONS

ADP	adenosine diphosphate
AQDS	anthraquinone-2,6-disulfonate
ATP	adenosine triphophate
CAS	chrome azurol S
DEFB	desferrioxamine B
DNA	deoxyrobose nucleic acid
DMRB	dissimilatory metal-reducing bacteria
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
EMS	ethyl methanesulfonate
FeCit	Fe(III)-citrate
FeRB	iron-reducing bacteria
gsp	general secretion pathway
HFO	hydrous ferric oxides (2L-ferrihydrite)
LB	Luria-Bertrani growth medium
MESA	micro-electrode screening array
MRB	metal-reducing bacteria
Mn(III)-pyro	Mn(III)-pyrophosphate
NAD(H)	nicotinamide adenine dinucleotide
NTA	nitrilotriacetic acid
ORF	open reading frame
PCR	polymerase chain reaction
QH_2	dibasic quinone
RNA	ribonucleic acid
S. oneidensis	*Shewanella oneidensis* MR-1

S. putrefaciens	*Shewanella putrefaciens* strain 200
SRB	sulfate-reducing bacteria
TEA	terminal electron acceptor
TMAO	trimethylamine oxide
Tris	tris(hydroxymethyl)aminomethane
UV-VIS	ultra violet- visible electromagnetic spectrum
w/v	weight per unit volume
v/v	volume per unit volume

SUMMARY

Microbial iron respiration contributes significantly to the biogeochemical cycling of metals and may be one of the earliest respiratory processes to have evolved on early earth. Metal-respiring microbes also hold great potential for use in microbial fuel cells for the generation of "green" energy and for remediation of radionuclides in contaminated environments. Despite its significance in global metal cycling processes, the molecular mechanism of Fe(III) respiration has yet to be determined. Unlike many other terminal electron acceptors, Fe(III) is a solid at circumneutral pH and, therefore, cannot come into direct contact with the microbial inner membrane: the site of terminal electron transfer in gram-negative bacteria. It is postulated that metal-respiring organisms have developed alternate strategies for the reduction of solid iron. One such strategy involves the production of an Fe(III)-solublizing ligand by the metal-respiring bacteria which solubilizes the Fe(III) prior to respiration, rendering the metal more easily accessible to the Fe(III) reductase complex.

In this study, the genes involved in the solubilization of Fe(III) by the gram-negative dissimilatory metal reducing bacteria *Shewanella oneidensis* MR-1 were determined using random mutagenesis to generate mutations in the wild-type genome and high-throughput square-wave voltammetry to screen for the attenuation of Fe(III) production in the mutants. Two mutants unable to solubilize Fe(III) were identified and designated d29 and d64. After mutation complementation analysis, it was determined that the point mutations were both located in type II secretion genes: *gspG* and *gspE*

respectively, indicating that the type II secretion system is required for Fe(III) solubilization prior to respiration.

It was also hypothesized that the ligand produced for Fe(III) solubilization during dissimilatory Fe(III) respiration was a siderophore: a small Fe(III)-chelating molecule produced by the cells for the assimilation of Fe(III) for growth. A siderophore biosynthesis gene (SO3031) and a siderophore ferric reductase gene (SO3034) were deleted in frame and the resultant mutants screened to determine whether they were capable of Fe(III) solubilization and reduction during anaerobic Fe(III) respiration. Both mutants retained Fe(III) solubilization and reduction activity, indicating that the siderophore Fe(III) assimilatory system is distinct from the Fe(III) solubilization system utilized during Fe(III) respiration.

The work presented here is significant in that it describes a rapid screening method for identifying Fe(III) solubilization mutants, reports on the involvement of the type II secretion system in Fe(III) solubilization during iron respiration, and finally demonstrates that a dissimilatory metal reducing bacteria synthesizes and secretes Fe(III)-chelating molecules which are distinct from Fe(III)-siderophores.

CHAPTER 1

INTRODUCTION

1.1 Abstract

Dissimilatory iron respiration is defined as energy generation via the electron transport chain using Fe(III) as terminal electron acceptor and a highly reduced molecule (such as lactate or H_2) as the initial electron donor. This process is globally significant as iron is the 4th most abundant element in the earth's crust and microbial iron respiration contributes greatly to the geochemical cycling of iron by both causing reductive dissolution of $Fe(III)_{(s)}$ to $Fe(II)_{(aq)}$ and by generating soluble Fe(III) from solid Fe(III). These processes have a significant effect on the environmental niches where these microbes are active, and are responsible for weathering of clays, oxidation of organic materials, and increasing the bioavailablity of iron in an ecosystem to name a few. Microbial metal respiration processes are also hypothesized to be some of the earliest forms of microbial energy generation to have occurred on early earth as indicated by the broad number of organisms capable of this form of respiration and by banded iron formations. Despite its obvious global significance, the molecular mechanism of microbial iron respiration has yet to be determined. Fe(III) is present in circumneutral environments as highly insoluble oxides or hydroxides, which cannot come into direct contact with the microbial inner membrane: the site of the electron transport chain in gram-negative bacteria. Therefore it is hypothesized that metal reducing bacteria must utilize a novel strategy for the effective respiration of solid Fe(III) compounds. The elucidation of this process will not only be enlightening from a purely intellectual

standpoint, but will also give considerable insight into the environmental processes and communities in which these organisms are active.

1.2 Significance of Dissimilatory Metal Reducing Bacteria

Environmental Impact

Dissimilatory metal reducing bacteria (DMRB) are integral components of redox stratified aquatic environments. Microbial metal respiration is primarily observed in redox stratified aquatic sediments or in water columns. In such systems, oxygen is rapidly consumed by the microbes residing in the upper layer of the water or sediment column, forcing microbes living in the lower layers into anaerobic respiration coupled to organic matter oxidation (Nealson and Myers, 1992; Roh et al., 2003) (Figure 1.1). Fe(III) reduction has been directly linked to organic decomposition in aquatic sediments (Rodin et al., 1996), decomposition of aromatic hydrocarbons (Lovley et al., 1995a), generation of high concentrations of dissolved iron in aquatic environments (Lovley et al., 1995b; Chapelle et al., 1995), and banded iron formation as a direct result of iron metabolism (Nealson, 1990). Metal-reducing bacteria are also closely associated with the weathering of clays and minerals in subsurface environments. Studies have demonstrated that magnetite and siderite formation are partly due to the biomineralization process of metal-reducing bacteria. These bacteria contribute greatly to the cycling of both iron and carbon (Nealson and Myers, 1992; Tortell et al., 1996; Roh et al., 2003). Fe and Mn solubilization is a result of respiration and fermentation by metal reducing bacteria. The resultant metal complexes formed are highly dependant on a variety of environmental factors, such as oxygen (or other constituent) availability (Nealson and Saffarini, 1994; Stumm and Morgan, 1996; Quantin et al., 2001). Weathering of silicates is occasionally

driven by the nutrient requirements of the microbial community that are dependent on the trace metal availability of the environment. Progression of mineral weathering is determined by the energetic value of the minerals available such that the minerals that provide the most energy to the cell, are destroyed most rapidly. Conversely, the population of species living in a community are determined by the minerals available and by the ability of the organism to make use of the available minerals (Bennett et al., 2001).

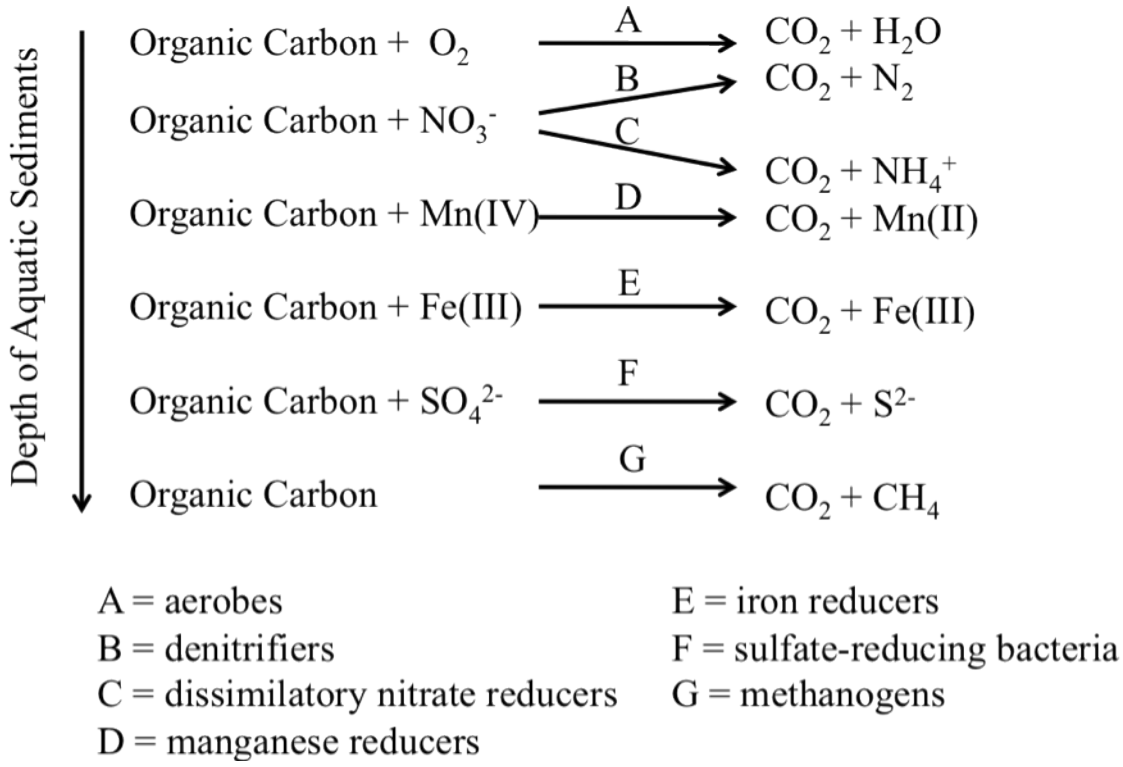

Figure 1.1 Redox stratification in aquatic sediments. Available terminal electron acceptors are ordered by redox potential.

Evolutionary Significance

Metal reducing organisms can be found in both the Archaeal and the Bacterial domains, including in a variety of proteobacteria subdivisions (Figure 1.2). The diversity of the organisms with this capacity apparently indicates that metal respiration may be deeply rooted in the tree of life and may be one of the earliest respiratory processes to have occurred on early earth (Pace, 1991; Holm, 1992).

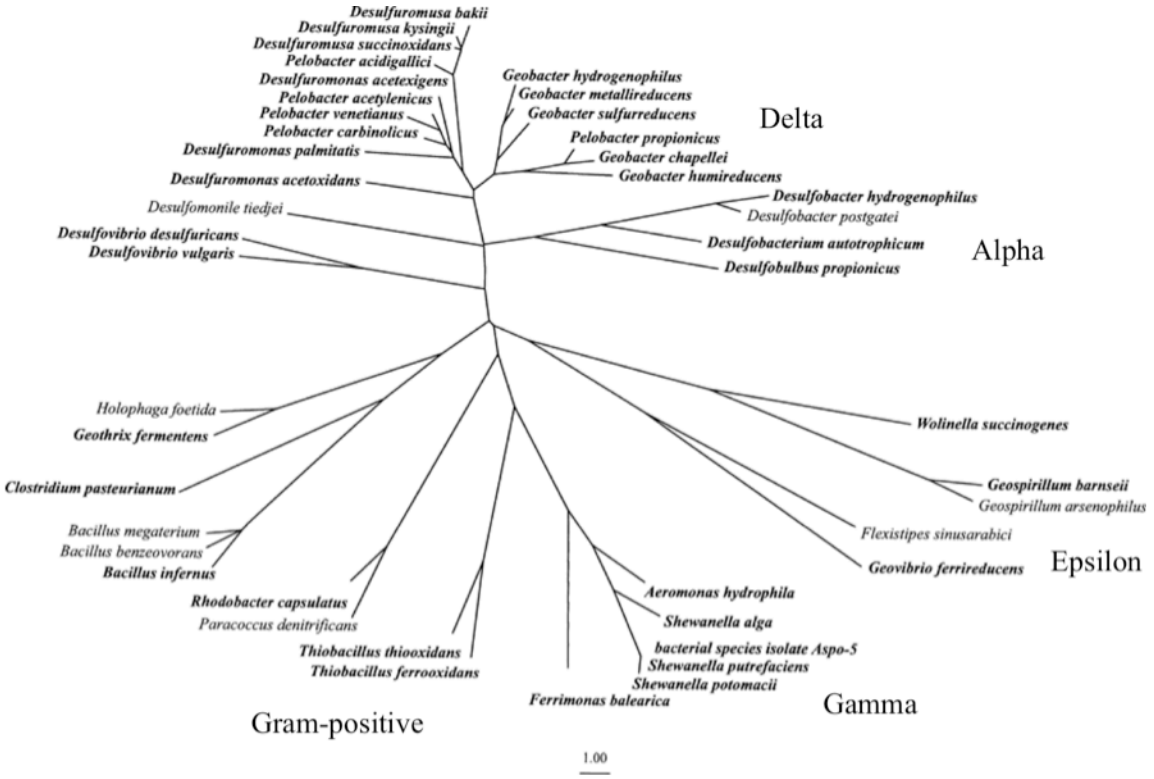

Figure 1.2 16S rRNA phylogenetic tree of the *Bacteria* domain depicting the diversity of iron reducing bacteria. Iron reducing bacteria are shown in bold; bar length represents 1 evolutionary distance unit. Adapted from (Lovley et al., 1997).

Evidence suggests that sulfur respiration in hydrothermal vents may have been the first respiratory process on early earth (Pace, 1991; Stetter, 1996), and studies of modern sulfur-reducing hyperthermophiles demonstrate that many are also capable of Fe(III) respiration (Vargas et al., 1998). Indeed, many iron-reducing bacteria have been identified in hyperthermophilic environments in the deep subsurface. Furthermore, Fe(III) was probably one of the most abundant terminal electron acceptors on early earth, arising from the photochemical oxidation of Fe(II) or from the oxidation of Fe(II) by anoxygenic phototrophs (Widdel et al., 1993; Kappler et al., 2005). Banded iron formations point to the deposition of Fe(III) via the oxidation of Fe(II) by microorganisms in the Precambrian era (Nealson and Myers, 1990) Terminal electron acceptors used by a large percentage of microorganisms today (such as oxygen, nitrite and sulfates) were most likely not present in significant concentrations in the reducing atmosphere of early earth, therefore these pockets of sedimented Fe(III) represented an energetically favorable electron acceptor for more recently evolved microorganisms.

Application in Microbial Fuel Cells

A potential application for such microorganisms may include their use in microbial fuel cells. Fuel cells have been suggested as a "green" alternative to traditional energy generation methods such as burning fossil fuels. In such cells, energy produced during respiration by metal reducing bacteria (MRBs) is harnessed and converted to electrical energy. Incubation of MRBs in the anodic chamber with both an electron donor and a carbon source (such as glucose) results in the generation of an electrical current as the MRBs transfer electrons to the cathode of the fuel cell, using it as a terminal electron acceptor. This electron transfer can occur in the presence or absence of

a mediator electron shuttle. Electrons passed from the cells during respiration are then harvested for electricity generation. (Liu and Logan, 2004; Logan et al., 2006; Du et al., 2007)

Application for Contaminant Radionuclide Remediation

Uranium and technetium are major groundwater contaminants in areas affected by radionuclide contaminations. Contaminated area is approximately 7280 km^2 total in 36 states and represents a significant threat to surrounding farmlands and waterways (Wall and Krumholz, 2006). Under oxic conditions, uranium is present in the form of uranyl oxide (UO_2^{2+}) and technetium is in the form of pertechnetate (TcO_4^-) which are water soluble and therefore highly mobile in subsurface aquifers. Reduction of U(VI) and Tc(VII) by metal-reducing microbes represents an attractive remediation strategy as the respiratory product (U(IV) is highly immobile at circumneutral pH, and therefore is less of a threat in subsurface aquifers (Valls and de Lorenzo, 2002; Gregory and Lovley, 2005). A wide variety of organisms are capable of radionuclide reduction, including *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Pyrobaculum*, *Thermus*, *Deinococcus*, *Clostridium*, *Desulfiosporosinus*, and *Shewanella* (Mohapatra et al., 2010). The ability of a particular organism to reduce these metals is dependant on the potential of the Tc or U species. U(VI) and Tc(VII) form complexes with organic ligands under circumneutral pH, and the reduction of such complexes when coupled to hydrogen or organic carbon oxidation is thermodynamically favorable. Organic or inorganic ligands bound to U(VI) affect uranium reduction and solubility; different organisms are best adapted to the reduction of different uranium forms which may indicate a variety of respiratory mechanisms (Ganesh et al., 1997). Reduction inhibition may also occur in the presence

of alternate terminal electron acceptors. This reductive precipitation process has been demonstrated to be effective in contaminated subsurface environments. Injection of exogenous electron donors resulted in burst of U(VI) reduction concomitant with Fe(III) reduction by FeRBs (Senko et al., 2002). Further results indicated that the electron donor used determines the community structure. Once U(VI) levels dropped below detectable levels, acetate-oxidizing sulfate-reducing bacteria dominated the community (Finneran et al., 2002).

1.3 Respiration in Gram-negative bacteria

General microbial respiration in gram-negative bacteria is a process in which energy in the form of adenosine-5'-triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH), which drive all other energy-requiring cellular processes, is produced via a series of electron transfer reactions though redox-active membrane-bound proteins and mobile redox active molecules. A variety of electron donor/accepter pairs can be coupled for energy generation, however the quantity of energy produced is dependent on the redox potential of both the electron donor and acceptor: the greater the difference in potential, the more energetically favorable the coupling. The redox poise of the donor and acceptor couple also determines which components of the electron transport chain are used. Dehydrogenase proteins (electron donor specific oxidoreductases) lie at the head of the chain and act as the primary oxidant to an electron donor, which is generally a molecule with a negative redox potential (such as H_2 or lactate). After the initial oxidation of the electron donor, the electrons are passed through a series of proteins that span a range of redox potentials including flavoproteins (enzymes with a covalently bound riboflavin moiety) which translocate protons across the inner

membrane during electron transfer, thus generating a proton gradient across the inner membrane, and iron-sulfur proteins (enzymes containing sulfide-linked di, tri or tetrairon centers) which transfer electrons to a quinone pool. Quinones (hydrophobic, non-protein-containing molecules with isoprene side chains which render them lipid soluble) are reduced on the inner aspect of the cytoplasmic membrane to QH_2, which is in turn translocated to the periplasm, increasing the concentration of protons in the periplasmic space. Electrons are subsequently passed from the quinone pool to a series of cytochromes (tetra-pyrole heme containing proteins). The electron transfer processes ultimately results in the transfer of electrons to a terminal electron acceptor (O_2 in the case of aerobic respiration). The proton gradient generated by the movement of electrons through the electron transport chain drives the production of ATP in the inner membrane localized protein ATP-synthase. As protons pass from the periplasm through the ATP-synthase to the cytoplasm, the enzyme catalyzes the phosphorylation of ADP to ATP (Figure 1.3) (Haddock and Jones, 1977; Trumpower and Gennis, 1994; Madigan et al., 2003).

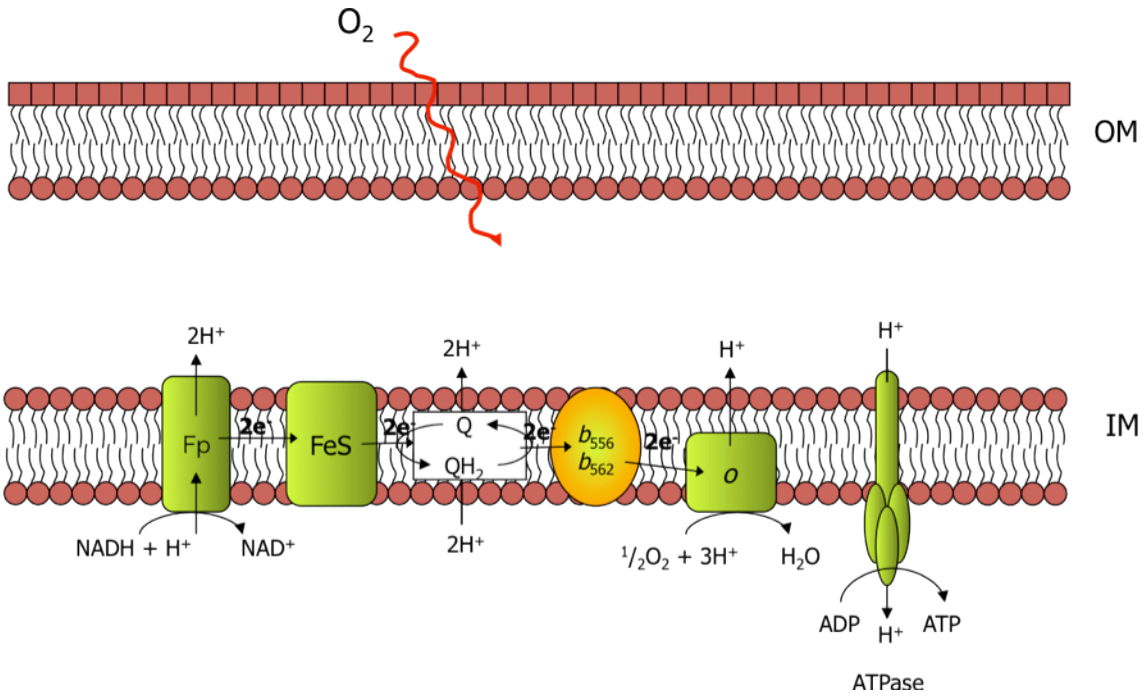

Figure 1.3 Model electron transport chain in gram-negative bacteria, adapted from (DiChristina et al., 2005).

1.4 *Shewanella*

The genus *Shewanella* is an ubiquitous gram-negative γ-proteobacterium which has been identified in a range of environments from the deep sea to oil brine to oyster habitats. It is characterized by its capability to use a broad range of organic (such as trimethylamine oxide (TMAO) and dimethylsulfoxide (DMSO)) and inorganic compounds (such as nitrates and intermediate sulfur compounds) as terminal electron acceptors. The genus has been of most interest because with few species exceptions, it is also capable of respiration on solid and soluble metals and some radioactive elements (such as uranium and technetium). Iron respiration occurs with the coupling of H_2 or organic acid oxidation to iron reduction in the following reactions:

$H_2 + 2Fe(III) \rightarrow 2H^+ + 2Fe(II)$

$CHOO^- + 2Fe(III) + H_2O \rightarrow HCO_3^- + 2Fe(II) + 2H^+$

$C_3H_6O_3 + 4Fe(III) + 2H_2O \rightarrow CH_3CO_2^- + HCO_3^- + 4Fe(II) + 5H^+$

$C_3H_2O_3 + 2Fe(III) + 2H_2O \rightarrow CH_3CO_2^- + HCO_3^- + 2Fe(II) + 3H^+$

Because it is a mesophilic, facultative anaerobe easily and quickly cultured in lab settings, it is an ideal model organism for studying Fe(III) respiration (Hau and Gralnick, 2007; Fredrickson et al., 2008).

1.5 Microbial Metal Respiration

The major obstacle encountered by iron-respiring organisms is that unlike soluble terminal electron acceptors (such as oxygen or nitrate), Fe(III)-oxides are solid at circumneutral pH and therefore cannot come into direct contact with the inner membrane of gram-negative bacteria (the site of terminal electron transfer to soluble electron acceptors). DRBs must therefore develop an alternate strategy for respiration of solid electron acceptors. Multiple strategies have been suggested including 1. the localization of the respiratory apparatus to the outer membrane for subsequent reduction of the metal via direct contact, 2. cycling of naturally occurring (such as humic acids) or endogenously produced (such as quinones) electron shuttling molecules) which transport electrons from the electron transport chain in the periplasm through the outer membrane to the iron oxides, 3. synthesis of electrically conductive appendages (termed nanowires) suggested to pass electrons though a series of heme-like proteins, and 4. synthesis and exudation of a Fe(III)-chelating molecules which solubilizes solid Fe(III) oxides and allows for respiration either in the periplasm or outer membrane. (DiChristina et al., 2005)

1.6 Soluble Fe(III)

Various studies have reported on the observation of soluble Fe(III) production in both natural and laboratory settings and its effect on dissimilatory metal reducers. Initial investigations demonstrate that the addition of the metal chelator nitrilotriacetic acid (NTA) to incubations of *Shewanella putrefaciens* strain 200 (formerly called *Pseudomonas* strain 200) with solid iron increased the rate of reduction 20-fold. Observed rates of reduction were found to correlate directly with the concentration of NTA added (Arnold et al., 1986). Later work demonstrates the correlation between rates of iron reduction by *S. putrefaciens* strain 200 and the type of organic molecule complexed to the Fe(III) atom. *S. putrefaciens* was cultivated on a suite of Fe(III)-organic complexes with thermodynamic stability complexes which span from a log*K* value of 4.4 to 25. Rates of reduction correlated to the stability constant of the Fe(III)-organic complex whereby the least stable complexes were most rapidly reduced (Haas and DiChristina, 2002). These results indicate that the solubility and speciation of the Fe(III) complex governs reduction rates by DMRBs. Soluble Fe(III) has been detected in natural environments. Gel fractionation of salt marsh porewater revealed the presence of Fe(II) and Fe(III)-organic complexes, presumably produced by DMRBs colonizing the porewater (Luther et al., 1996).

Laboratory studies of pure cultures of DMRBs demonstrate production of soluble Fe(III) complexes when cells are incubated with solid Fe(III). Incubations of *Geothrix fermentans* (a DMRB) with solid iron revealed that cultures produced compounds which stimulated Fe(III) reduction. Poorly crystalline Fe(III) was placed in microporous beads with *Geothrix* (such that the cells were prevented from coming into direct contact with

the Fe(III) and both Fe(II) and soluble Fe(III) was detected via ion chromatography (Nevin and Lovley, 2002b). Similarly, incubations of *Shewanella alga* sp. BrY with Fe(III)-oxides within microporous beads yielded 450 μM dissolved Fe(III) in the medium (Nevin and Lovley, 2002a). Later studies demonstrated that Fe(III) complexes are detected during *Shewanella putrefaciens* sp. 200 reduction of Fe(III) (as either Fe(III)-oxides or Fe(III)-citrate) proportional to the reactivity of Fe(III) phase and cell density. Abiotic Fe(III)-citrate does not display a signal when screened via square-wave voltammetry, yet when incubated with *Shewanella*, soluble Fe(III) (defined as organic complexes of Fe(III) which are detected at mercury electrodes in the potential range of -0.3V to -0.06) is observed indicating that the organic ligand produced by the DMRB destabilizes the Fe(III)-citrate complex. This is the first example of a voltammetric technique being applied to detect soluble Fe(III) (Taillefert et al., 2000; Taillefert et al., 2007).

1.7 Type II Secretion and OmcA/MtrC

Previous studies have demonstrated the importance of the type II protein secretion system (a general secretory pathway typically used to secreted proteins onto the outer membrane) on metal respiration in MRBs such as *Shewanella* (Figure 1.4). *S. putrefaciens* strain 200 mutations containing an insertional mutation in *ferE*, a homologous gene to the type II protein secretion gene *gspE*, were unable to respire Fe(III) or Mn(IV), yet retained the ability to respire all other terminal electron acceptors tested. Subsequent analysis revealed a 91kD metal-reducing protein complex located on the outer membrane of wild-type cells which was absent in the *ferE* type II secretion mutant (DiChristina et al., 2002). A homologous metal-reducing complex was found on

the outer membrane of *S. oneidensis* MR-1, and two decaheme *c*-type cytochromes (MtrC and OmcA) were identified within the complex. In-frame deletion mutants of *gspD* (the porin of the secretion system) and *gspG* (the rapidly polymerizing pseudopilus of the secretion system which pushes the target protein onto the outer membrane) did not display either MtrC or OmcA on their outer surface, indicating that the type II secretion system is directly involved in the translocation of these cytochromes to the cell surface. (Shi et al., 2008).

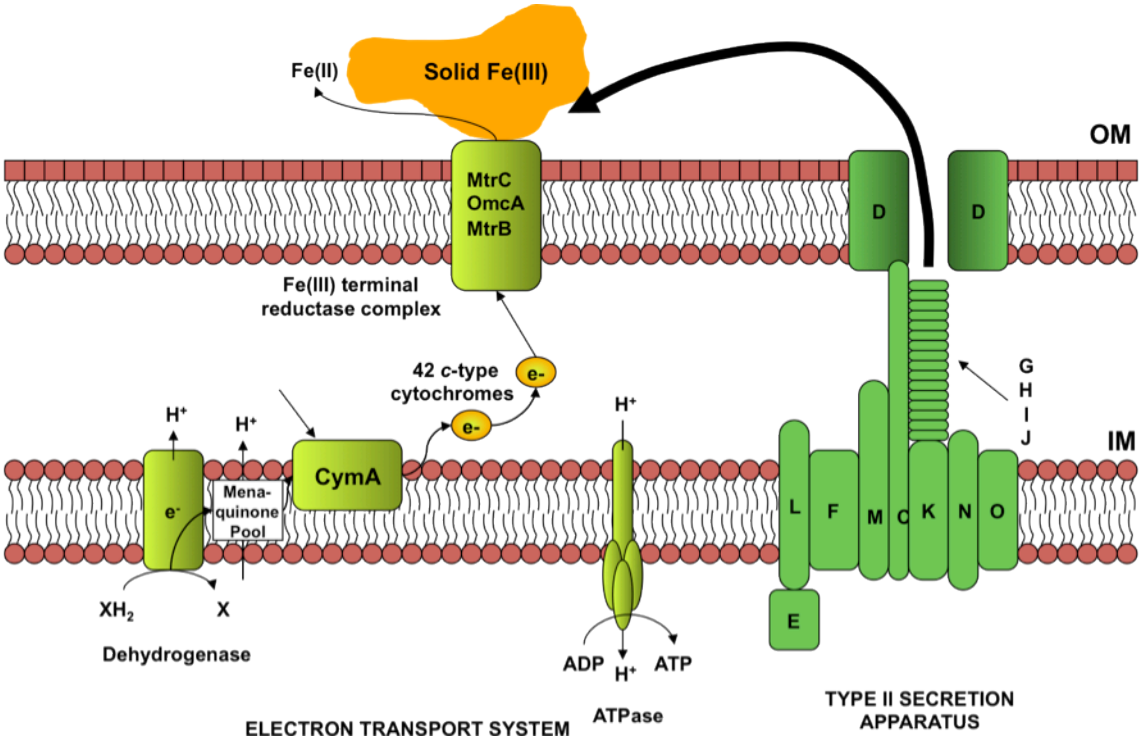

Figure 1.4 Postulated electron transport chain with terminal metal reductase localized on the outer membrane by the type II secretion system. Depicted with solid Fe(III) as terminal electron acceptor. Adapted from (DiChristina et al., 2002)

It was therefore hypothesized that either of these two cytochromes act as the terminal metal reductase involved in dissimilatory metal respiration. Corroboration for this hypothesis was obtained in further studies. Membrane fractions from wild-type *S. oneidensis* MR-1 and a corresponding MtrC mutant were tested for Fe(III)-reduction capabilities. Wild-type fractions were able to reduce Fe(III), yet fractions from the MtrC deletion mutant exhibited decreased Fe(III)-reduction activity (Beliaev et al., 2001). Further, the heme groups from purified MtrC from *S. oneidensis* MR-1 were identified as low spin, magnetically coupled heme groups spanning a redox potential of +100 - -500 mV, falling within the redox range for Fe(III) reduction (Hartshorne et al., 2007). Further work on both MtrC and OmcA seemed to indicate that the two cytochromes work in concert as a terminal metal reductase. Atomic force microscopy experiments with purified protein of both *c*-type cytochromes revealed specific bonding to hematite surface: OmcA demonstrates stronger interactions with the hematite, but MtrC binding is more favorable, however both are capable of electron transfer to the hematite surface (as measured via cyclic voltammetry) (Lower et al., 2007; Meitl et al., 2009). Single-molecule analysis revealed that while both cytochromes have similar biochemical and molecular properties, their electron tunneling properties are quite different. (Wigginton et al., 2007). Data from these two experiments suggest that a cooperative action of the two cytochromes is necessary. Additionally, in protein binding experiments, MtrC was demonstrated to copurifiy with OmcA. And while each individual cytochrome was capable of Fe(III) (in the form of Fe(III)-NTA) reduction, enhanced rates of reduction were observed when both cytochromes were present (Shi et al., 2006).

Recently however, the newly adopted dogma that metal reduction requires direct contact has been called into question, however. Kinetic experiments demonstrated that direct contact of MtrC and OmcA alone cannot account for the rates of Fe(III) reduction observed with whole cell incubations with solid Fe(III). When soluble Fe(III) was incubated with purified MtrC and OmcA, the observed rates of reduction were comparable to those predicted by a direct-contact model. However, when the two cytochromes were incubated with solid Fe(III), the rates observed were much slower than those observed in whole cell incubations with solid Fe(III). This apparently indicates that a mediator molecule is involved and that direct contact alone is not sufficient for metal respiration. (Ross et al., 2009). Further doubt was cast on the hypothesis of direct contact for respiration when it was determined that a maximum distance of 15Å between the outer membrane cytochromes and the iron oxide surface must be achieved for electron transfer to occur (Neal et al., 2005). Although this distance is achievable (allowing for conformational adjustment of the cytochromes on the mineral oxide surface), the electron transfer step would become a significant bottleneck in the bioreduction process. Additionally, rates of electron transfer were also highly sensitive to heme configuration, donor-acceptor distance, porphyrin orientation, surface termination and possible intervening water molecules (Kerisit et al., 2007). Thus, although the *c*-type cytochromes MtrC and OmcA are undoubtedly crucial to metal reduction in *S. oneidensis* MR-1, it seems unlikely that they directly mediate the terminal electron transfer to solid metals.

1.8 Siderophores

Shewanella species, along with a broad range of other fungi and bacteria, produce siderophores, Fe(III)-chelating molecules produced for the purpose of iron assimilation. Representative siderophores are depicted in Figure 1.5. Siderophores are low molecular weight complexes synthesized by the cell and secreted into the extracellular milieu where they chelate one or more Fe(III) atoms. The Fe(III)-chelate complex is subsequently taken-up by the cell where the Fe(III) is reduced to Fe(II) and incorporated into a variety of iron containing proteins such as iron-sulfur proteins. Siderophore biosynthesis is regulated by the Fur system: when the cell is iron starved, siderophore biosynthesis genes are expressed. When the cell is iron replete, a Fur-Fe complex represses gene expression and siderophore biosynthesis is halted (Raymond et al., 1984; Wandersman and Delepelaire, 2004). Siderophore biosynthesis occurs via a non-ribsomal peptide synthesis (NRPS) machinery. Each module of the NRPS assembly line contains a carrier protein domain with an HS arm that tethers the growing siderophore chain. The other portions of each module are catalytic and carry out local enzyme chemistry on the growing chain (such as methylation or cyclization) (Finking and Marahiel, 2004). The basic monomer used is usually species-specific and determines the structure and specific binding of the siderophore to Fe(III), though the most common monomers are hydroxamate or catecholate containing molecules as they have a high binding affinity to Fe(III), though carboxylic acids may also be used. These monomers range from molecules synthesized by the cell (such as salicylate or dihydroxybenzoate) whose biosynthesis genes closely regulated with the NRPS expression, or they constitute an amino acid (such as serine or threonine) which is already readily available in the cell. (Crosa and Walsh, 2002). Just

as the siderophores produced are species-specific, so are the outer membrane receptor proteins. The receptors identify siderophores based on siderophore structure and chemistry and the complex is carried across the inner and outer membranes in an ATP dependent, TonB mediated process.

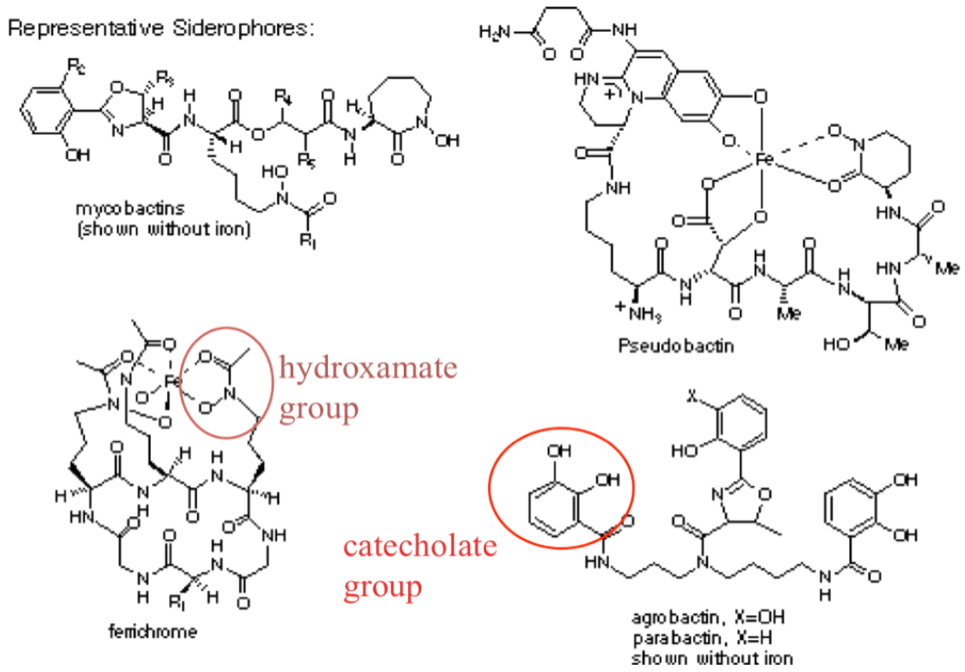

Figure 1.5 Representative siderophores from various bacteria: mycobactin from *Mycobaterium*, pseudobactin from *Pseudomonas*, ferrichrome found in multiple bacterial species, agrobactin from *Agrobacterium* and parabactin from *Paracoccus.*

Further siderophore specificity is achieved by adding additional functional groups to the monomer backbone or via cyclization reactions. In the case of *Shewanella*, the dominant siderophore produced is a cyclic dimer of succinyl (N-hydroxy-putrecine) called putrebactin which is synthesized from ornithine monomers and is similar in structure to alcaligin and bisucaberin which are hydroxamate-type siderophore utilized by unrelated bacterial species. (Ledyard and Butler, 1997).

1.9 RESEARCH OBJECTIVES

The main objective of this research was to identify the genes involved in Fe(III) solubilization during Fe(III) respiration by *Shewanella oneidensis* MR-1. Despite the extensive research conducted in the field of microbial metal respiration, the molecular mechanisms involved remain unclear. Preliminary data indicate that *Shewanella* generates soluble Fe(III) under Fe(III) respiratory conditions, but the genes and proteins involved in this process remained unidentified. To go about identifying these genes, two major experimental strategies were followed: 1. generation of random point mutants unable to solubilize Fe(III) during anaerobic Fe(III) respiration and subsequent genetic complementation to identify the genes involved in the solubilization process and 2. in-frame deletion of known ferric siderophore biosynthesis and reduction genes and subsequent screening for Fe(III)-solubilization and growth with Fe(III) as a terminal electron acceptor.

1.10 References

Arnold, R.G., DiChristina, T.J., and Hoffmann, M.R. (1986) Inhibitor studies of dissimilative Fe(III) reduction by *Pseudomonas* sp. strain 200 ("*Pseudomonas ferrireductans*"). *Applied and Environmental Microbiology* **52**: 281-289.

Beliaev, A.S., Saffarini, D.A., McLaughlin, J.L., and Hunnicutt, D. (2001) MtrC, an outer membrane decahaem *c* cytochrome required for metal reduction in *Shewanella putrefaciens* MR-1. *Molecular Microbiology* **39**: 722-730.

Bennett, P.C., Rogers, J.R., and Choi, W.J. (2001) Silicates, silicate weathering, and microbial ecology. *Geomicrobiology Journal* **18**: 3-19.

Chapelle, F.H., McMachon, P.B., and Dubrovsky, N.M. (1995) Deducing the distribution of terminal electron-accepting processes in the hydrologically diverse groundwater systems. *Water Resources Research.* **31**: 359-371.

Crosa, J.H., and Walsh, C.T. (2002) Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiology and Molecular Biology Reviews* **66**: 223-249.

DiChristina, T.J., Moore, C.M., and Haller, C.A. (2002) Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires *ferE*, a homolog of the *pulE* (*gspE*) type II protein secretion gene. *Journal of Bacteriology* **184**: 142-151.

DiChristina, T.J., Fredrickson, J.K., and Zachara, J.M. (2005) Enzymology of electron transport: energy generation with geochemical consequences. *Reviews in Mineralogy and Geochemistry* **59**: 27-52.

Du, Z.W., Li, H.R., and Gu, T.Y. (2007) A state of the art review on microbial fuel cells: A promising technology for wastewater treatment and bioenergy. *Biotechnology Advances* **25**: 464-482.

Finking, R., and Marahiel, M.A. (2004) Biosynthesis of nonribosomal peptides. *Annual Review of Microbiology* **58**: 453-488.

Finneran, K.T., Anderson, R.T., Nevin, K.P., and Lovley, D.R. (2002) Potential for ioremediation of uranium-contaminated aquifers with microbial U(VI) reduction. *Soil & Sediment Contamination* **11**: 339-357.

Fredrickson, J.K., Romine, M.F., Beliaev, A.S., Auchtung, J.M., Driscoll, M.E., Gardner, T.S. et al. (2008) Towards environmental systems biology of *Shewanella*. *Nature Reviews Microbiology* **6**: 592-603.

Ganesh, R., Robinson, K.G., Reed, G.D., and Sayler, G.S. (1997) Reduction of hexavalent uranium from organic complexes by sulfate- and iron-reducing bacteria. *Applied and Environmental Microbiology* **63**: 4385-4391.

Gregory, K.B., and Lovley, D.R. (2005) Remediation and recovery of uranium from contaminated subsurface environments with electrodes. *Environmental Science & Technology* **39**: 8943-8947.

Haas, J.R., and DiChristina, T.J. (2002) Effects of Fe(III) chemical speciation on dissimilatory Fe(III) reduction by *Shewanella putrefaciens*. *Environmental Science and Technology* **63**: 373-380.

Haddock, B.A., and Jones, C.W. (1977) Bacterial respiration. *Bacteriological Reviews* **41**: 47-99.

Hartshorne, R.S., Jepson, B.N., Clarke, T.A., Field, S.J., Fredrickson, J., Zachara, J. et al. (2007) Characterization of *Shewanella oneidensis* MtrC: a cell-surface decaheme cytochrome involved in respiratory electron transport to extracellular electron acceptors. *Journal of Biological Inorganic Chemistry* **12**: 1083-1094.

Hau, H.H., and Gralnick, J.A. (2007) Ecology and biotechnology of the genus *Shewanella*. *Annual Review of Microbiology* **61**: 237-258.

Holm, N.G. (1992) Why are hydrothermal systems proposed as plausible environments for the origin of life. *Origins of Life and Evolution of the Biosphere* **22**: 5-14.

Kappler, A., Pasquero, C., Konhauser, K.O., and Newman, D.K. (2005) Deposition of banded iron formations by anoxygenic phototrophic Fe(II)-oxidizing bacteria. *Geology* **33**: 865-868.

Kerisit, S., Rosso, K.M., Dupuis, M., and Valiev, M. (2007) Molecular computational investigation of electron-transfer kinetics across cytochrome-iron oxide interfaces. *Journal of Physical Chemistry C* **111**: 11363-11375.

Ledyard, K.M., and Butler, A. (1997) Structure of putrebactin, a new dihydroxamate siderophore produced by *Shewanella putrefaciens*. *Journal of Biological Inorganic Chemistry* **2**: 93-97.

Liu, H., and Logan, B.E. (2004) Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. *Environmental Science & Technology* **38**: 4040-4046.

Logan, B.E., Hamelers, B., Rozendal, R., Schrorder, U., Keller, J., Freguia, S. et al. (2006) Microbial fuel cells: methodology and technology. *Environmental Science & Technology* **40**: 5181-5192.

Lovley, D.R., and Anderson, R.T. (1995a) Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface. *Hydrogeology Journal* **8**: 77-88.

Lovley, D.R., and Chapelle, F.R. (1995b) Deep subsurface microbial processes. *Reviews of Geophysics* **33**: 365-381.

Lovley, D.R., Coates, J.D., Saffarini, D., and Lonergan, D.J. (1997) Dissimilatory Fe(III) reduction. In *Transition metals in microbial metabolism*. Winkelman, G., and Carrano, C.J. (eds). Amesteldjik, The Netherlands: Harwood Academic Publishers, pp. 187-219.

Lower, B.H., Shi, L., Yongsunthon, R., Droubay, T.C., McCready, D.E., and Lower, S.K. (2007) Specific bonds between an iron oxide surface and outer membrane cytochromes MtrC and OmcA from *Shewanella oneidensis* MR-1. *Journal of Bacteriology* **189**: 4944-4952.

Luther, G.W., Shellenbarger, P.A., and Brendel, P.J. (1995) Dissolved organic Fe(III) and Fe(II) complexes in salt marsh porewaters. *Geochemica et Cosmochimica Acta* **60**: 951-960

Madigan, M., Martinko, J., and Parker, J. (2003) *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Prentice Hall/Pearson Education.

Meitl, L.A., Eggleston, C.M., Colberg, P.J.S., Khare, N., Reardon, C.L., and Shi, L. (2009) Electrochemical interaction of *Shewanella oneidensis* MR-1 and its outer membrane cytochromes OmcA and MtrC with hematite electrodes. *Geochimica Et Cosmochimica Acta* **73**: 5292-5307.

Mohapatra, B.R., Dinaro, O., Gould, W.D., and Koren, D.W. (2010) Biochemical and genomic facets on the dissimilatory reduction of radionuclides by microorganisms - A review. *Minerals Engineering* **23**: 591-599.

Neal, A.L., Bank, T.L., Hochella, M.F., and Rosso, K.M. (2005) Cell adhesion of *Shewanella oneidensis* to iron oxide minerals: Effect of different single crystal faces. *Geochemical Transactions* **6**: 77-84.

Nealson, K.H., and Myers, C.R. (1990) Iron Reduction by Bacteria - a Potential Role in the Genesis of Banded Iron Formations. *American Journal of Science* **290A**: 35-45.

Nealson, K.H., and Myers, C.R. (1992) Microbial reduction of manganese and iron: new approaches to carbon cycling. *Applied and Environmental Microbiology* **58**: 439-443.

Nealson, K.H., and Saffarini, D. (1994) Iron and Manganese in Anaerobic Respiration - Environmental Significance, Physiology, and Regulation. *Annual Review of Microbiology* **48**: 311-343.

Nevin, K.P., and Lovley, D.R. (2002a) Mechanisms for Fe(III) oxide reduction in sedimentary environments. *Geomicrobiology Journal* **19**: 141-159.

Nevin, K.P., and Lovley, D.R. (2002b) Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. *Applied and Environmental Microbiology* **68**: 2294-2299.

Pace, N.R. (1991) Origin of life - Facing up to the physical setting. *Cell* **65**: 531-533.

Quantin, C., Becquer, T., Rouiller, J.H., and Berthelin, J. (2001) Oxide weathering and trace metal release by bacterial reduction in a New Caledonia Ferralsol. *Biogeochemistry* **53**: 323-340.

Raymond, K.N., Muller, G., and Matzanke, B.F. (1984) Complexation of iron by siderophores - A review of their solution and structural chemistry and biological function. *Topics in Current Chemistry* **123**: 49-102.

Roden, E.E., and Wetzel, R.G. (1996) Organic carbon oxidation and suppression of methane production by microbial Fe(III) oxide reduction in vegetated and unvegetated freshwater wetland sediments. *Limnology and Oceanography* **41**: 1733-1748.

Roh, Y., Zhang, C.L., Vali, H., Lauf, R.J., Zhou, J., and Phelps, T.J. (2003) Biogeochemical and environmental factors in Fe biomineralization: Magnetite and siderite formation. *Clays and Clay Minerals* **51**: 83-95.

Ross, D.E., Brantley, S.L., and Tien, M. (2009) Kinetic characterization of OmcA and MtrC, terminal reductases involved in respiratory electron transfer for dissimilatory iron reduction in *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* **75**: 5218-5226.

Senko, J.M., Istok, J.D., Suflita, J.M., and Krumholz, L.R. (2002) In-situ evidence for uranium immobilization and remobilization. *Environmental Science & Technology* **36**: 1491-1496.

Shi, L., Deng, S., Marshall, M.J., Wang, Z.M., Kennedy, D.W., Dohnalkova, A.C. et al. (2008) Direct involvement of type II secretion system in extracellular translocation of *Shewanella oneidensis* outer membrane cytochromes MtrC and OmcA. *Journal of Bacteriology* **190**: 5512-5516.

Shi, L., Chen, B.W., Wang, Z.M., Elias, D.A., Mayer, M.U., Gorby, Y.A. et al. (2006) Isolation of a high-affinity functional protein complex between OmcA and MtrC: Two outer membrane decaheme *c*-type cytochromes of *Shewanella oneidensis* MR-1. *Journal of Bacteriology* **188**: 4705-4714.

Stetter, K.O. (1996) Hyperthermophilic procaryotes. *Fems Microbiology Reviews* **18**: 149-158.

Stumm, W., and Morgan, J.J. (1996) *Aquatic Chemistry*. New York: John Wiley and Sons, Inc.

Taillefert, M., Luther, G.W., and Nuzzio, D.B. (2000) The application of electrochemical tools for *in situ* measurements in aquatic sediments. *Electroanalysis* **12**: 401-412.

Taillefert, M., Beckler, J.S., Carey, E.A., Burns, J.L., Fennessey, C.M., and DiChristina, T.J. (2007) *Shewanella putrefaciens* produces an Fe(III)-solubilizing ligand during anaerobic respiration on insoluble Fe(III) oxides *Journal of Inorganic Biochemistry* **101**: 1760-1767.

Tortell, P.D., Maldonado, M.T., and Price, N.M. (1996) The role of heterotrophic bacteria in iron-limited ocean ecosystems. *Nature* **383**: 330-332.

Trumpower, B.L., and Gennis, R.B. (1994) Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration - The enzymology of coupling electron-transfer reactions to transmembrane proton translocation. *Annual Review of Biochemistry* **63**: 675-716.

Valls, M., and de Lorenzo, V. (2002) Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *Fems Microbiology Reviews* **26**: 327-338.

Vargas, M., Kashefi, K., Blunt-Harris, E.L., and Lovley, D.R. (1998) Microbiological evidence for Fe(III) reduction on early Earth. *Nature* **395**: 65-67.

Wall, J.D., and Krumholz, L.R. (2006) Uranium reduction. *Annual Review of Microbiology* **60**: 149-166.

Wandersman, C., and Delepelaire, P. (2004) Bacterial iron sources: From siderophores to hemophores. *Annual Review of Microbiology* **58**: 611-647.

Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B., and Schink, B. (1993) Ferrous iron oxidation by anoxygenic phototrophic bacteria. *Nature* **362**: 834-836.

Wigginton, N.S., Rosso, K.M., Lower, B.H., Shi, L., and Hochella, M.F. (2007) Electron tunneling properties of outer-membrane decaheme cytochromes from *Shewanella oneidensis*. *Geochimica Et Cosmochimica Acta* **71**: 543-555.

CHAPTER 2

SHEWANELLA ONEIDENSIS MR-1 MUTANTS SELECTED FOR THEIR INABILITY TO PRODUCE SOLUBLE ORGANIC-FE(III) ARE UNABLE TO RESPIRE FE(III) AS AN ANAEROBIC ELECTRON ACCEPTOR

2.1 Abstract

Recent voltammetric analyses indicate that *Shewanella putrefaciens* strain 200 produces soluble organic-Fe(III) during anaerobic respiration of sparingly soluble Fe(III) oxides. Results of the present study expand the range of *Shewanella* species capable of producing soluble organic-Fe(III) to include *S. oneidensis* MR-1. Soluble organic-Fe(III) was produced by *S. oneidensis* cultures incubated anaerobically with Fe(III) oxides, or with Fe(III) oxides and the alternate electron acceptor fumarate, but not in the presence of O_2, nitrate or trimethylamine-*N*-oxide. Chemical mutagenesis procedures were combined with a novel MicroElectrode Screening Array (MESA) to identify four (designated Sol) mutants with impaired ability to produce soluble organic-Fe(III) during anaerobic respiration of Fe(III) oxides. Two of the Sol mutants were deficient in anaerobic growth on both soluble Fe(III)-citrate and Fe(III) oxide, yet retained the ability to grow on a suite of seven alternate electron acceptors. The rates of soluble organic-Fe(III) production were proportional to the rates of iron reduction by the *S. oneidensis* wild-type and Sol mutant strains, and all four Sol mutants retained wild-type siderophore production capability. Results of this study indicate that the production of soluble organic-Fe(III) may be an important intermediate step in the anaerobic respiration of both soluble and sparingly soluble forms of Fe(III) by *S. oneidensis*.

2.2 Introduction

Dissimilatory iron reducing bacteria (DIRB) impact a variety of important environmental processes, including biogeochemical cycling of carbon and iron, bioremediation of organic and inorganic contaminants, and generation of electricity in microbial fuel cells (Nealson and Saffarini, 1994; Lovley et al., 2004; Logan et al., 2006; Gralnick and Newman, 2007). SSU rRNA analyses indicate that DIRB are deeply rooted and scattered throughout the domains *Archaea* and *Bacteria* (Weber et al., 2006), an indication that dissimilatory iron reduction may have been one of the first respiratory processes to have evolved on early earth (Vargas et al., 1998; Schulze-Makuch et al., 2005). DIRB play integral roles in remineralization of organic matter in redox-stratified, aqueous environments (Thamdrup, 2000; Kostka et al., 2002; Koretsky et al., 2003), and in remobilization of iron in iron-limited oceans (Elrod et al., 2004; Severmann et al., 2006; Gerringa et al., 2007). Some DIRB degrade hazardous organics in contaminated ground water (Lovley et al., 1989; Zachara et al., 1998) or work in concert with fermenting bacteria to decompose recalcitrant organic compounds (Watson et al., 2005). Metal, metalloid, and radionuclide solubility is also altered by DIRB activity in natural and contaminated environments (Lovley et al., 1991; DiChristina et al., 2005) and DIRB-catalyzed, reductive precipitation (immobilization) of radionuclides has been proposed as a alternate remediation strategy in radionuclide-contaminated environments.

At circumneutral pH, Fe(III) is sparingly soluble and is typically found in the form of crystalline and poorly ordered oxyhydroxides (Zinder et al., 1986). Consequently, neutrophilic DIRB are postulated to employ a variety of novel respiratory pathways not found in other bacteria that respire soluble electron acceptors such as O_2,

NO_3^-, SO_4^{2-}, and CO_2 (DiChristina et al., 2005; Weber et al., 2006). Several alternate pathways for respiration of sparingly soluble Fe(III) oxides have been proposed, including: 1) a direct enzymatic pathway in which terminal Fe(III) reductases, localized on the outer membrane or on electroactive nanowires, contact and deliver electrons directly to the Fe(III) oxide surface (Myers and Myers, 1992; DiChristina et al., 2002; Reguera et al., 2005; Gorby et al., 2006; Shi et al., 2006); 2) a two-step electron shuttling pathway in which endogenous or exogenous electron shuttles are first reduced by DIRB followed by chemical reduction of the Fe(III) oxide by the reduced electron shuttle in a second (abiotic) electron transfer reaction (Newman and Kolter, 2000; Coates et al., 2002; Hernandez et al., 2004; Marsili et al., 2008); and 3) a two-step Fe(III) chelation (solubilization) pathway in which solid Fe(III) oxides are first non-reductively dissolved by endogenous organic ligands prior to the DIRB-catalyzed reduction of the soluble organic-Fe(III) complexes (Arnold et al., 1988; Lovley and Woodward, 1996; Taillefert et al., 2002). Cells adjacent to the mineral surface are postulated to employ the direct enzymatic pathways, while cells at a distance may rely on the electron shuttling or Fe(III) solubilization pathways. For the latter two pathways, the energetic cost of synthesizing extracellular, electron transfer components must be offset by faster reduction rates, increased energy production, or increased access to electron acceptors (Taillefert et al., 2007; Marsili et al., 2008). Thus, the recycling of Fe(III)-chelating compounds or electron shuttles between the cell and mineral surfaces may minimize the energetic costs associated with DIRB biosynthesis and provide advantages over other respiratory pathways.

Evidence for Fe(III) solubilization in the environment includes the voltammetric detection of high concentrations of soluble organic-Fe(III) in porewaters of redox stratified sediments (Brendel and Luther, 1995; Taillefert et al., 2000b; Taillefert et al., 2002). Voltammetric analyses demonstrate that soluble organic-Fe(III) is readily formed at circumneutral pH in both laboratory and field experiments, an indication that Fe(III) solubilization may be an important first step in the microbial reduction of Fe(III) oxides (Taillefert et al., 2000a; Carey and Taillefert, 2005; Crowe et al., 2007; Marsili et al., 2008). In laboratory cultures, soluble organic-Fe(III) enhances the rate and/or extent of DIRB-catalyzed Fe(III) reduction (Arnold et al., 1988; Dollhopf et al., 2000; Nevin and Lovley, 2002; Taillefert et al., 2007). Soluble organic-Fe(III) is also reduced more rapidly than Fe(III) oxides by purified outer membrane *c*-type cytochromes of the gram-negative DIRB *Shewanella oneidensis* MR-1, a metal respiring member of the γ-proteobacteria (Myers and Myers, 2003; Shi et al., 2006; Xiong et al., 2006). As the outer membrane *c*-type cytochromes of *Shewanella* are postulated to act as terminal Fe(III) reductases during anaerobic Fe(III) respiration (DiChristina et al., 2005; Fredrickson and Zachara, 2008), the production of soluble organic-Fe(III) may therefore enhance the overall Fe(III) respiratory activity of *Shewanella* (Taillefert et al., 2007).

In addition to soluble and sparingly soluble forms of Fe(III), *S. oneidensis* also respires a wide variety of alternate terminal electron acceptors, including oxygen (O_2), nitrate (NO_3^-), nitrite (NO_2^-), manganese oxides (Mn(III,IV)), trimethylamine-*N*-oxide (TMAO), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), elemental sulfur (S(0)), uranium [U(VI)], technetium(Tc(VII)), fumarate, anthraquinone 2,6-disulphonate (AQDS), and potentially several others (Myers and Nealson, 1988; Nealson and Saffarini, 1994; Venkateswaran et

al., 1999; Wade and DiChristina, 2000; Payne and DiChristina, 2006). The suite of electron acceptors respired by *S. oneidensis* spans nearly the entire range of redox potentials encountered in the environment. Recent voltammetric analyses have demonstrated that *S. putrefaciens* strain 200 produces soluble organic-Fe(III) during anaerobic respiration of Fe(III) oxides as electron acceptor (Taillefert et al., 2007). The production of soluble organic-Fe(III) during anaerobic Fe(III) respiration by *S. oneidensis* has yet to be examined.

The main objectives of the present study were to 1) determine if *S. oneidensis* was capable of producing soluble organic-Fe(III) during anaerobic respiration of Fe(III) oxides, 2) develop a voltammetric-based, mutant screening technique for rapid identification of *S. oneidensis* (designated Sol) mutants unable to produce soluble organic-Fe(III) during anaerobic respiration of Fe(III) oxides, 3) test the Sol mutants for siderophore production activity and for anaerobic growth on a variety of alternate, anaerobic electron acceptors, including soluble and sparingly soluble forms of Fe(III), and 4) compare the rates of soluble organic Fe(III) production to the rates of Fe(II) production to determine if soluble organic Fe(III) production is a potential rate-limiting step in the overall Fe(III) respiratory activity of *S. oneidensis*.

2.3 Materials and Methods

Bacterial strains, cultivation conditions and mutagenesis procedures

S. oneidensis MR-1 (ATCC no. 700550) was originally isolated from Oneida Lake, NY (Myers and Nealson, 1988). Anaerobic respiratory mutant strain T121 (only capable of aerobic growth) isolated via transposon mutagenesis of *S. putrefaciens* 200R (Saffarini et al., 1994) was used as a negative control strain for anaerobic respiration. All

Shewanella strains were cultured on previously described *Shewanella* growth medium (DiChristina and Delong, 1994).

Previously described chemical (ethyl methanesulfonate; EMS) mutagenesis procedures were used to generate a set of randomly mutagenized *S. oneidensis* strains (DiChristina and Delong, 1994; Burnes et al., 1998; Taratus et al., 2000; Wade and DiChristina, 2000; Payne and DiChristina, 2006). Liquid cultures of MR-1 were grown aerobically to late log phase, harvested by centrifugation, washed, and suspended in PM-2 buffer (70 mM Na_2HPO_4, 30 mM NaH_2PO_4, 1 mM $MgSO_4$, and 200 μM $MnSO_4$). EMS was added to a final concentration of 19 μL mL^{-1}, and the cell suspension was incubated with gentle mixing for 1 hour to achieve 90% kill. Surviving cells were plated on LB agar and incubated aerobically for 1 day at 30 °C. A total of 3840 colonies arising from EMS-mutagenized cells were subsequently tested for soluble organic-Fe(III) production during anaerobic respiration of 2L-ferrihydrite.

Preparation of 2L-ferrihydrite

All solutions were prepared with ACS or trace metal grade chemicals in autoclaved 18 MΩ-water (Milli-Q). 2L-ferrihydrite was prepared by dissolving 54 g of $FeCl_3 \cdot 6H_20$ into 500 mL sterile H_20. Fe(III) oxides were precipitated by raising the pH to 7.0 by the addition of 10 N NaOH (Schwertmann and Cornell, 2000). The resulting 0.4 M mixture was stored in the dark until needed. Just before use, the desired volume was centrifuged and the supernatant decanted. The precipitated iron oxides were then washed three times with sterile H_2O and used as a 0.4 M suspension of 2L-ferrihydrite.

Construction of a Microelectrode Screening Array (MESA)

This work was conducted by Morris E. Jones.

A Microelectrode Screening Array (MESA) for voltammetric detection of soluble organic-Fe(III) complexes was constructed to screen large numbers of mutants in the individual 300 µL wells of a 96-well tray (8 x 12 matrix). MESA consists of an electrode holder with eight removable 100 µm diameter gold-mercury (Au/Hg) amalgam working electrodes and eight permanent 0.5 mm diameter Ag/AgCl reference and 0.5 mm diameter platinum counter electrodes mounted on a manual micromanipulator (Narishige). A machined aluminum tray holder with raised stops corresponding to the distance between the rows of the 96-well trays facilitated deployment of the tray from one row to another for screening (Figure 2.2A). The array of eight counter electrodes (Figure 2.2B) was fabricated by soldering eight one inch pieces of 0.5 mm platinum wire together every 0.8 cm along a 8 cm piece of striped stranded coaxial cable such that the distance between each wire was equal to the distance between the wells of a 300 µL 96-well tray. A similar array of reference electrodes was fabricated from 0.5 mm silver wire. The counter and reference arrays were placed 0.7 cm apart in a 12 cm x 1 cm x 2 cm mold such that 2 cm of the unstripped coaxial cables were within the mold and 2 cm of the wires were outside the mold. The mold was filled with epoxy resin and eight 5/32 inch holes were drilled between the counter and reference to receive the 3/16 inch PEEK™ tubing sheath of the working electrodes. Tapped holes were added to secure working electrodes with set screws. The Ag/AgCl reference electrodes were conditioned by applying +9 volts for 30 seconds between the reference and counter electrodes in a 3 M KCl solution. Au/Hg working electrodes were fabricated by soldering a one inch

length of 100 μm gold wire to the end of stranded coaxial cable. The gold wire and exposed copper wire were threaded into a 1.0 mm outer diameter glass tube. The joint between the glass tube and coaxial cable was covered by a one inch piece of 3/16 inch PEEK™ tubing sheath for reinforcement and mounting in the array body. The glass end was filled with epoxy until excess epoxy was observed from all joints. After drying, the tip was sanded with 400 grit sandpaper. BNC connectors were added and electrodes were polished, plated and conditioned as previously described (Brendel and Luther, 1995). Microelectrodes were first tested for quality using O_2, then calibrated with Mn^{2+}. Minor differences in electrode construction, polishing, and mercury plating generally result in electrodes that vary in mercury size, geometry, and analyte sensitivity (Osteryoung and Osteryoung, 1985; Brendel and Luther, 1995). Current intensities of each electrode were therefore normalized by multiplying the measured analyte intensity by the current intensity ratio of a Mn^{2+} standard for a given electrode to the average Mn^{2+} current intensity for the eight electrodes in the array (Eq. 2.1):

$$I_{normalized} = I_{measured} \cdot \frac{I_{standard}}{average(I_{standard})} \tag{2.1}$$

where $I_{normalized}$ is the normalized current intensity, $I_{measured}$ is the measured current intensity of the analyte of interest, $I_{standard}$ is the current intensity of a 200 μM Mn^{2+} standard, and $average(I_{standard})$ is the average current intensity of the Mn^{2+} standard for the eight electrodes of the array. A Model DLK-100 potentiostat with DLK-MUX-1 eight channel electrode multiplexer (Analytical Instruments Systems, Inc.) was used for all voltammetric measurements. Working electrodes were controlled by the computer-operated DLK-MUX-1 multiplexer, while the reference and counter electrodes shared respective leads between each cell and the DLK-100A potentiostat. All voltammetric

potentials are reported versus the Ag/AgCl reference electrode.

Detection of *S. oneidensis* (designated Sol) mutants unable to produce soluble organic-Fe(III)

Single colonies arising from EMS-mutagenized cells were transferred to individual wells of a 96-well tray containing 32 mM 2L-ferrihydrite in *Shewanella* growth medium. Each tray included one row of wild-type *S. oneidensis* and an abiotic control row with cells omitted. Inoculated trays were incubated in a Coy anaerobic chamber (atmosphere consisting of 85% N_2, 10% CO_2, 5% H_2) at 30 °C for 30-36 hrs prior to voltammetric analysis. Each well of a row contained an individual working electrode and shared reference and counter electrodes. MESA was lowered into a row of the 96-well tray and the sequence started to automatically measure voltammetric signals across the individual wells of a row. At the end of a sequence, MESA was lifted, the tray moved to the next stop of the base plate, and MESA was lowered into the next row of eight wells. This procedure was repeated for each of the 12 rows of a tray. Analysis time for all 96 wells was approximately 2 hours. Voltammetric conditions used in triplicate measurements included: a conditioning step for 10 sec at -0.9 V to reduce any Fe(III) species on the electrode surface prior to a subsequent measurement, a deposition step for 10 sec at -0.1 V to concentrate Fe(III) species at the electrode surface, a scan rate of 200 mV s^{-1} from -0.1 to -1.8 V to cover the ranges of Fe(III) and Fe(II) reduction potentials, and a pulse height of 0.05 V (Taillefert et al., 2000b). Voltammograms were integrated using the program VOLTINT in Matlab™ (Bristow and Taillefert, 2008). Mutant colonies displaying aberrant voltammetric responses were rescreened in triplicate and

subsequently confirmed for Sol mutant phenotypes in individual batch reactor incubations.

Confirmation of Sol mutant phenotypes in batch reactor incubations

Wild-type *S. oneidensis* MR-1 and the putative Sol mutant strains were incubated in 100 mL PEEK™ batch reactors containing *Shewanella* growth medium supplemented with 32 mM 2L-ferrihydrite as electron acceptor and 20 mM sodium lactate as electron donor. Reactors were inoculated with cells at a final concentration of 2 x 10^7 cells mL^{-1} and incubated in a Coy chamber under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2) with gentle stirring. For experiments with competing electron acceptors, O_2 was introduced via constant bubbling with compressed air, or 15 mM nitrate, 25 mM trimethylamine oxide (TMAO), or 10 mM fumarate were added from filter-sterilized stock solutions. Two 200 µL aliquots were removed every six to eight hours for ancillary analyses. The first 200 µL aliquot was added to 1 mL 0.5 M HCl to extract adsorbed Fe^{2+} (Lovley and Phillips, 1986) and analyzed for total Fe^{2+} via the Ferrozine colorimetric method (Stookey, 1970). The second was transferred to a well of a 300 µL 96-well tray for voltammetric analysis as described above. Initial rates of organic-Fe(III) production were determined by linear regression of the increase in organic-Fe(III) current intensities for 24 hours (disregarding any initial lag period). Initial rates of Fe(III) reduction were determined by linear regression of the total Fe(II) production rate during the period of soluble organic-Fe(III) production. All rates were normalized to cellular protein content determined via previously described procedures(DiChristina and Delong, 1994).

Siderophore detection with Chrome Azurol S (CAS)

Siderophores were detected during growth on liquid or solid *Shewanella* growth media via application of CAS-based techniques. CAS screening plates were prepared using a modified version of a previously described procedure (Schwyn and Neilands, 1987). Blue CAS (Sigma Aldrich) agar was prepared by adding 60.5 mg of CAS dye dissolved in 50 mL water to 10mL acidic solution of $FeCl_3$ (1 mM $FeCl_3$, 10 mM HCl). This mixture was slowly added to a solution of surfactant hexadecyltrimethyl amine (HDTMA) (Sigma Aldrich) (72.9 mg dissolved in 40 mL H2O), and the resulting solution was autoclaved, cooled to 55ºC and added to 900 mL sterile *Shewanella* growth media supplemented with 1.5% w/v agar (CAS agar). CAS shuttling solution was prepared as previously described (Schwyn and Neilands, 1987). Siderophore production by *S. oneidensis* wild-type and Sol mutants was monitored by patching colonies onto CAS agar plates, incubating aerobically for 24 hours or anaerobically for 48 hours (atmosphere of 85% N_2, 10% CO_2, 5% H_2), and visually scoring the colony periphery for yellow halos. Siderophore production was also monitored during aerobic growth in liquid *Shewanella* growth medium with lactate as electron donor. Liquid culture aliquots of 0.5 mL were centrifuged for 1 min (12,000 *g*), and the resulting supernatant was mixed with 0.5 mL CAS shuttling solution and allowed to incubate for 3 hours. Samples were subsequently measured spectrophotometrically at 630 nm to determine siderophore production. Absorbance readings are recorded as the absorbance of the CAS-treated sample divided by the absorbance of CAS-treated uninnoculated growth medium.

Determination of overall respiratory capability of *S. oneidensis* and Sol mutants

S. oneidensis wild-type and Sol mutants were inoculated in liquid *Shewanella* growth medium (final concentration of 10^7 cells mL^{-1}) amended with either 18 mM lactate or 60 mM formate as electron donor and either saturated O_2, 15 mM nitrate, 50 mM dimethylsulfoxide (DMSO), 25 mM trimethylamine-*N*-oxide (TMAO), 10 mM fumarate, 10 mM thiosulfate, 50 mM Fe(III)-citrate, or 20 mM 2L-ferrihydrite as electron acceptor. For growth with O_2 as electron acceptor, compressed air was vigorously bubbled through the flasks. Anaerobic conditions were maintained by continuous sparging with N_2 (g). Cell growth was monitored by absorbance measurements at 600 nm over time. Nitrite (NO_2^-) concentrations in nitrate-grown cultures, were monitored by 250-fold aliquot dilution in a solution consisting of 9.6 mM sulfanilic acid, 96 mM $KHSO_4$, and 3.2 mM N,N-ethylenediamine (Montgomery and Dymock, 1962). Samples were held in the dark for 15 minutes prior to absorbance measurements at 510 nm. Fe(III) reduction was determined from total Fe(II) production over time monitored by the Ferrozine technique (Stookey, 1970) after extraction with HCl (Lovley and Phillips, 1986). Cell growth was monitored by direct cell counts via epifluorescence microscopy. Acridine orange-stained cells were counted (Carl Zeiss AxioImager Z1 Microscope) according to previously described procedures (Lovley and Phillips, 1988).

2.4 Results

Soluble organic-Fe(III) was produced by *S. oneidensis* liquid cultures incubated anaerobically with 2L-ferrihydrite, or with 2L-ferrihyrite and the alternate electron acceptor fumarate, but not in the presence of O_2, NO_3^-, or TMAO (Figure 2.1).

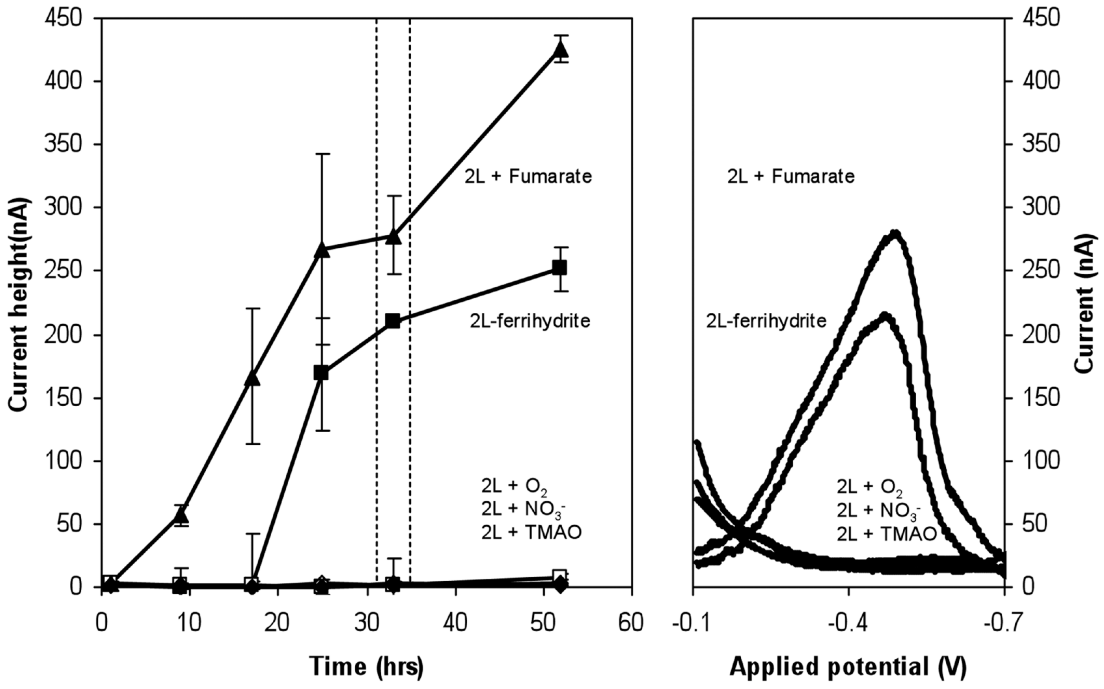

Figure 2.1 Production of soluble organic-Fe(III) by wild-type *S. oneidensis* incubated anaerobically with lactate as electron donor and 2L-ferrihydrite as electron acceptor or with 2L-ferrihydrite in the presence of alternate electron acceptors O_2, NO_3^-, TMAO, and fumarate. 2L-ferrihydrite (■), O_2 + 2L-ferrihydrite (□), NO_3^-,+ 2L-ferrihydrite (◆), TMAO + 2L-ferrihydrite (◇), fumarate + 2L-ferrihydrite (▲).

Anaerobic abiotic control incubations indicate that soluble organic-Fe(III) was not produced by interaction with lactate or any other component of the *Shewanella* growth medium (data not shown). Voltammetric signals indicative of soluble organic-Fe(III) were detected after an initial lag phase (16 h) and reached a maxima of 252 ± 17 nA for

2L-ferrihydrite alone. In turn, the incubation containing 2L-ferrihydrite and the alternate electron acceptor fumarate produced a soluble organic-Fe(III) signal after 8 hours which reached a maximum of 425 ± 10 nA at 52 hours. The rates of soluble organic-Fe(III) production in incubations of 2L-ferrihydrite with fumarate and the 2L-ferrihydrite alone were not significantly different, suggesting that the same solubilization process was involved in these incubations. No voltammetric signal was observed after 52 hours in incubations containing 2L-ferrihydrite and the alternate electron acceptors O_2, NO_3^-, or TMAO.

A mercury-gold (Hg/Au) MicroElectrode Screening Array (MESA) was constructed to identify EMS-mutagenized strains of *S. oneidensis* for their inability to produce soluble organic-Fe(III) during anaerobic incubation with 2L-ferrihydrite (Figure 2.2).

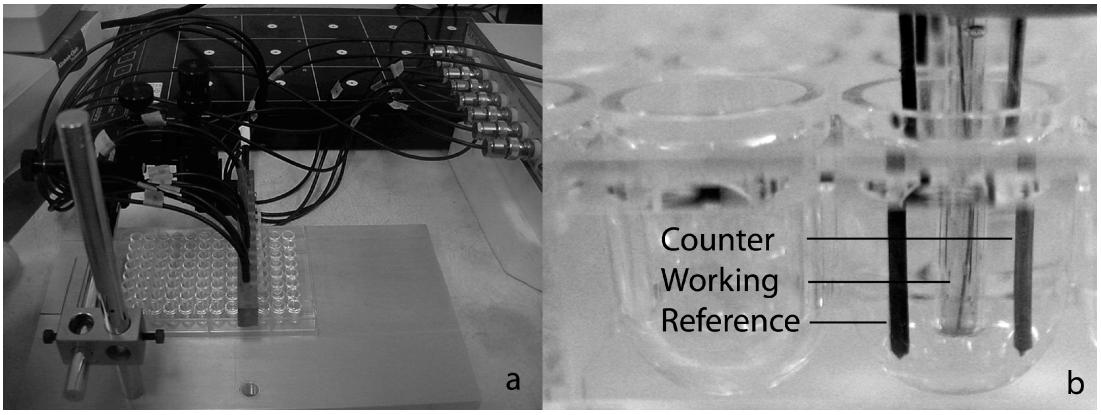

Figure 2.2 Microelectrode screening array (MESA) developed to identify Sol mutants. An array of eight Au/Hg voltammetric microelectrodes was used to analyze each row of a 96-well tray. A computer-controlled multiplexer automatically switched between working electrodes during each analysis. (A) View of MESA in a 96-well tray. (B) Interconnected platinum counter (left) and Ag/AgCl reference (right) electrodes are fixed within the resin body, while Au/Hg working electrodes (center) can be removed for polishing and storage.

The voltammograms resulting from wild-type *S. oneidensis* cultures incubated in 96-well

trays were identical to those observed with single electrode systems for dissolved oxygen and Mn^{2+} standards in 0.25 M NaCl (data not shown). Initially, rows of EMS-mutagenized strains were alternated with rows of 0.25 M NaCl blanks to wash the electrodes and check for inadvertent transfer of cells between rows. Control experiments with wild-type *S. oneidensis* indicated that inadvertent transfer was not a problem (data not shown), and the washing step was omitted in subsequent Sol mutant screening experiments. The first row of a tray contained wild-type *S. oneidensis* to ensure that conditions for wild-type organic-Fe(III) production were maintained during the 36 h anaerobic incubation period. The next row of the tray contained *Shewanella* growth medium with cells omitted (abiotic control). Finally, the last ten rows of the tray contained mutagenized cells.

A total of 3840 EMS mutants were screened via MESA for the ability to produce soluble organic-Fe(III). Four putative Sol mutants (designated d29, d64, H1, and B7) were identified with impaired soluble organic-Fe(III) production activity (defined as <50% wild-type activity) (Figure 2.3B).

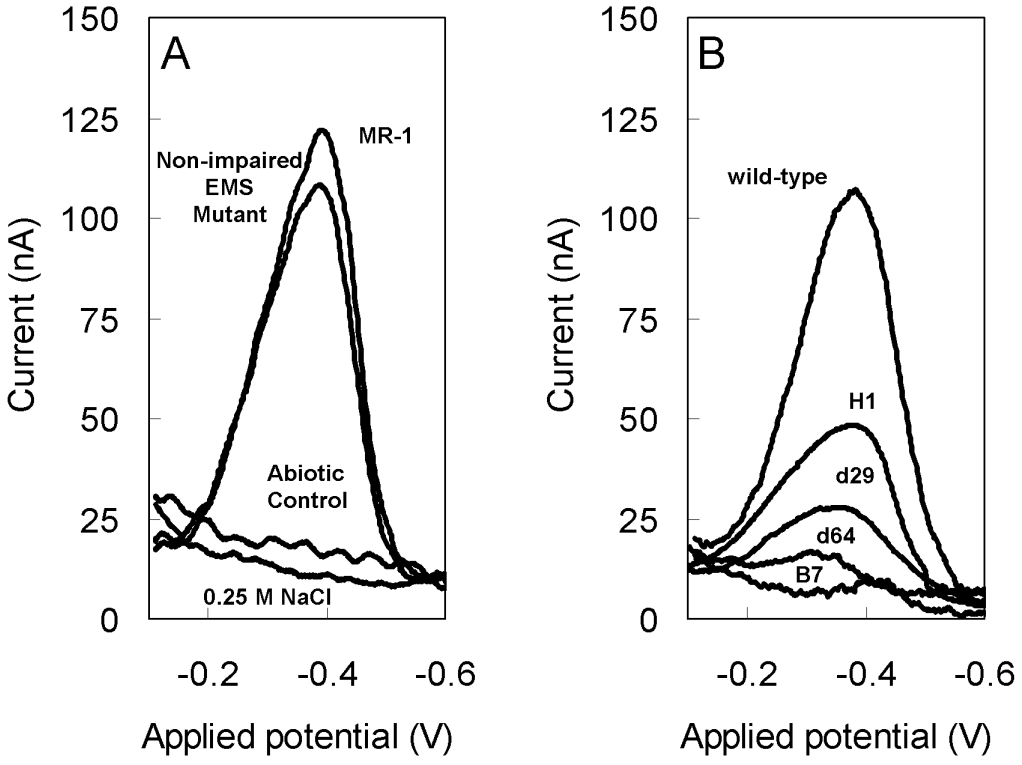

Figure 2.3 Voltammetric signals obtained from liquid cultures of wild-type *S. oneidensis* and a randomly selected, non-impaired EMS mutant (A) and Sol mutants d29, d64, H1, and B7 (B) incubated anaerobically in *Shewanella* growth medium supplemented with lactate as electron donor and 2L-ferrrihydrite as electron acceptor.

Wild-type *S. oneidensis* produced current intensities of 88.2 (± 31.9) nA, while the 3836 non-impaired, EMS-mutagenized strains produced current intensities of 102.7 (± 34.2) nA (abiotic controls produced current intensities <2% of wild-type *S. oneidensis*) (Table 2.1).

Table 2.1 Average current intensities of soluble organic-Fe(III) production measured by MESA in the presence of wild-type *S. oneidensis* and Sol mutants d29, d64, H1, and B7 compared to other non-impaired EMS mutants

Sample	Current (nA)*	% wild-type	S.D.**	n
Wild-type MR-1	88.2	-	31.9	384
Abiotic control***	1.9	2%	3.4	384
Sol mutant d29	24.2	27%	4.3	5
Sol mutant d64	14.6	17%	1.8	5
Sol mutant H1	40.4	46%	5.0	5
Sol mutant B7	9.0	10%	1.6	5
All other non-impaired EMS mutants****	102.7	116%	34.2	3836

*Current intensities were normalized to a 200 μM Mn(II) standard.
**Standard deviations of the average current intensities are reported for n measurements.
*** Incubation of 2L-ferrihydrite with cells omitted.
**** Average current intensities of the remaining 3,836 mutants screened.

Current intensities increased with time as cultures were in the growth phase. As a consequence, non-impaired mutants, analyzed last during screening, generally produced higher current intensities than wild-type *S. oneidensis*.

To confirm the Sol mutant phenotype, each of the four Sol mutants was incubated individually in *Shewanella* growth medium amended with 2L-ferrihydrite. Soluble organic-Fe(III) was observed in wild-type *S. oneidensis* incubations by 18 hours and reach steady state value of 350 nA after 60 hours (Figure 2.4A).

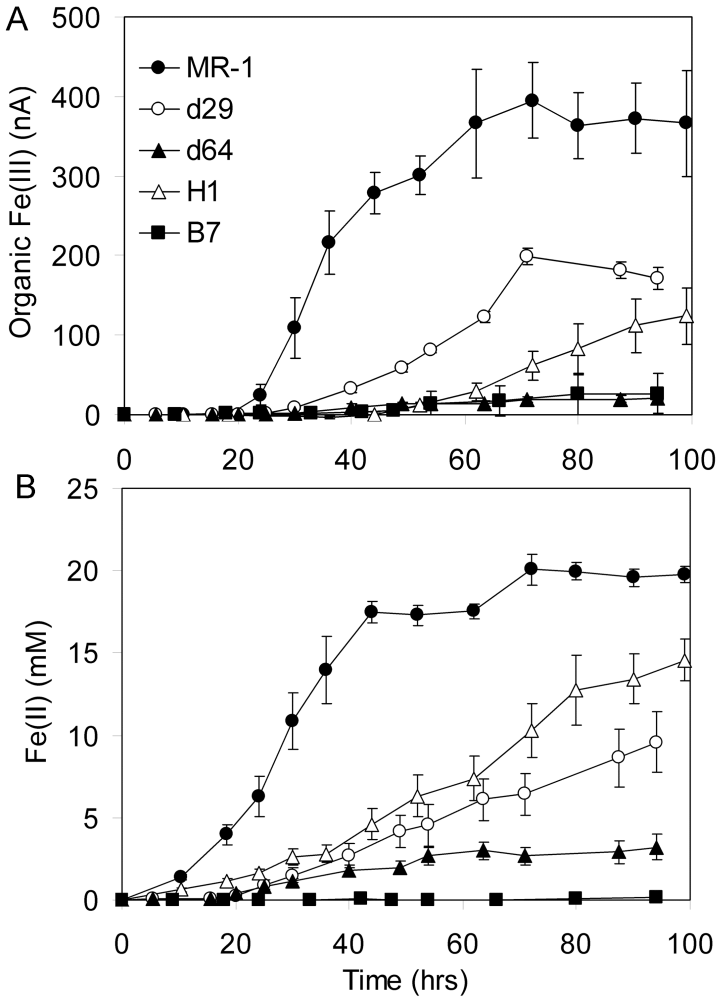

Figure 2.4 Anaerobic incubations of wild-type *S. oneidensis* (MR-1) and Sol mutants d29, d64, H1, and B7 in batch reactors amended with lactate as electron donor and 2L-ferrihydrite as electron acceptor: production of soluble organic-Fe(III) (A) and total Fe(II) (B) as a function of time. Error bars represent standard deviations calculated from three parallel yet independent anaerobic incubations. *S. oneidensis* MR-1 (●) and Sol mutants: d29 (○), d64 (▲), H1, (△), and B7 (■).

The Sol mutants d29 and H1 produced organic-Fe(III) signals late in the experiment reaching maximum current intensities of 200 nA and 125 nA. No significant organic-Fe(III) signals were produced by the Sol mutants d64 or B7. Fe(III) reduction in wild-type *S. oneidensis* incubations reach a maximum of 20 mM Fe(II) (Figure 2.4B). Sol

mutants d29, H1, and d64 produced maximums of 10 mM, 14 mM, and 2.5 mM Fe(II). The Sol mutant B7 produced no Fe(II) over the course of the experiment.

Wild-type *S. oneidensis* produced soluble organic-Fe(III) at an initial rate of 21.5 nA L mg protein^{-1} hr^{-1}, while all Sol mutants produced soluble organic Fe(III) at rates lower than the wild-type strain (% of wild-type rate in parentheses): d29 (35%), d64 (3%), H1 (25%), and B7 (1%) (Table 2.2).

Table 2.2 Rates of production of soluble organic-Fe(III) and Fe(II) by *S. oneidensis* wild-type and Sol mutants

	Soluble organic-Fe(III) production		Fe(II) production	
Strain	Rate (nA L mg protein^{-1} hr^{-1})	% wild-type	Rate (mmol mg protein^{-1} hr^{-1})	% wild-type
Wild-type MR-1	21.5 ± 3.5	-	855 ± 74	-
Sol mutant d29	7.5 ± 1.9	35 ± 11	275 ± 28	32 ± 4
Sol mutant d64	0.6 ± 0.2	3 ± 1.6	78 ± 20	9 ± 2.5
Sol mutant H1	5.3 ± 2.9	25 ± 15	500 ± 114	58 ± 14
Sol mutant B7	0.2 ± 0.1	1 ± 1.0	13 ± 5	1 ± 0.4

Wild-type *S. oneidensis* produced Fe(II) at a rate of 855 mmol mg protein^{-1} hr^{-1}, while all Sol mutants produced Fe(II) at rates lower than the wild-type strain (% of wild-type rate in parentheses): d29 (32%), d64 (9%), H1 (58%), and B7 (1%) (Table 2.2). The initial rates of Fe(III) reduction and organic-Fe(III) production were highly correlated with a R^2 greater than 0.8 and a slope of 24 (± 4) nA organic-Fe(III) mM^{-1} Fe(II) (Figure 2.5).

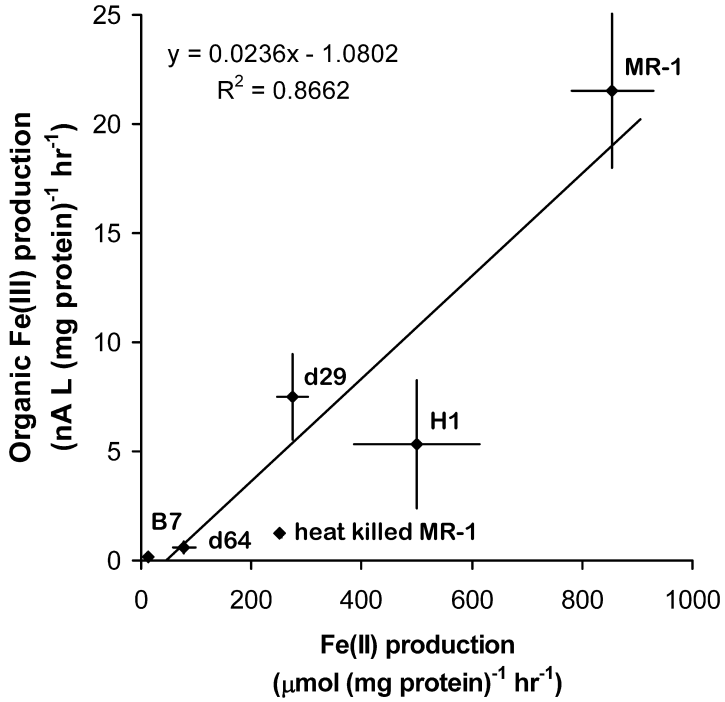

Figure 2.5 Correlation between initial rates of organic-Fe(III) production and initial rates of Fe(III) reduction by wild-type *S. oneidensis* and Sol mutants d29, d64, H1, and B7 incubated anaerobically with lactate as electron donor and 2L-ferrihydrite as electron acceptor.

The overall respiratory activity of the EMS mutants was tested on combinations of two electron donors and nine electron acceptors (Table 2.3).

Table 2.3 Respiratory capabilities* of *S. oneidensis* wild-type, anaerobic respiratory mutant T121, and Sol mutants with impaired organic-FeIII) production activity

	O_2		NO_3^-		NO_2^-		DMSO		TMAO		Fumarate		$S_2O_3^{2-}$		Fe(III)-Cit		Fe(III)-Ox	
Strains	L	F	L	F	L	F	L	F	L	F	L	F	L	F	L	F	L	F
Wild-type MR-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T121	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sol mutant d29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Sol mutant d64	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Sol mutant H1	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Sol mutant B7	+	+	+	+	+	+	-	-	-	+	-	-	+	+	-	-	-	-

*Respiratory capability: +, >50% wild-type growth rate; -, <50% wild-type growth rate
Electron donors: L = lactate, F = formate; Electron acceptors: O_2 = oxygen, NO_3^- = nitrate, NO_2^- = nitrite, DMSO = dimethyl sulfoxide, TMAO = trimethylamine oxide, $S_2O_3^{2-}$ = thiosulfate, Fe(III)-Cit = ferric citrate, Fe(III)-Ox = 2L-ferrihydrite.

Sol mutants d29 and d64 grew at wild-type rates on O_2, NO_3^-, NO_2^-, DMSO, TMAO, fumarate, and $S_2O_3^{2-}$ as electron acceptor (S1), yet were unable to grow at wild-type rates on either 2L-ferrihydrite (Figure 2.6) or Fe(III) citrate (Figure 2.7), regardless of electron donor.

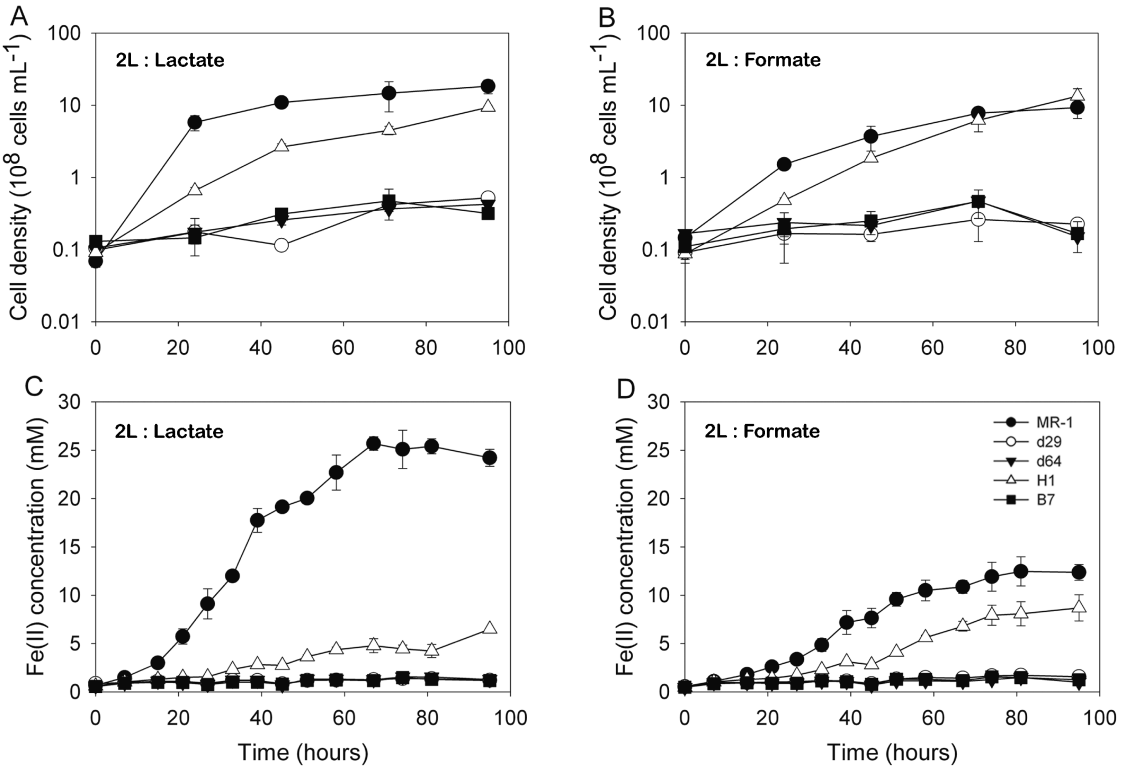

Figure 2.6 Anaerobic incubations of wild-type *S. oneidensis* (MR-1) and Sol mutants d29, d64, H1, and B7 in batch reactors amended with lactate or formate as electron donor and 2L-ferrihydrite as electron acceptor: cell density as a function of time with lactate (A) or formate (B) as electron donor, Fe(II) concentration as a function of time with lactate (C) or formate (D) as electron donor. *S. oneidensis* MR-1 (●) and Sol mutants: d29 (○), d64 (▲), H1, (△), and B7 (■).

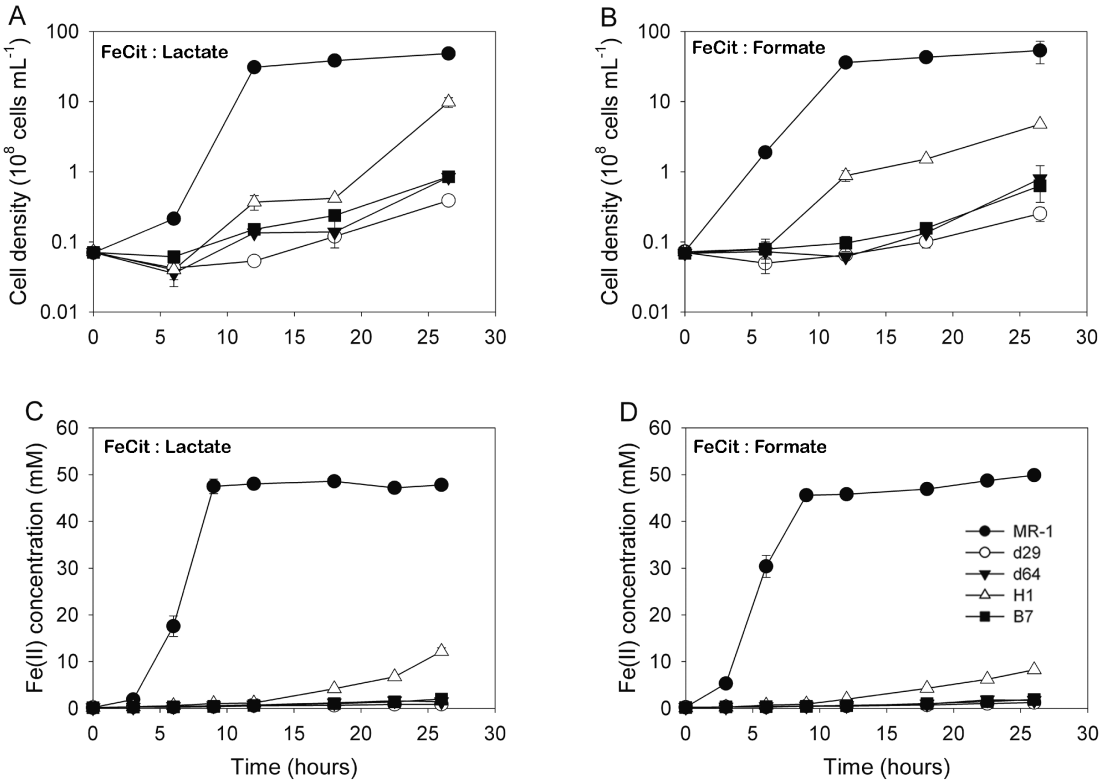

Figure 2.7 Anaerobic incubations of wild-type *S. oneidensis* (MR-1) and Sol mutants d29, d64, H1, and B7 in batch reactors amended with lactate or formate as electron donor and Fe(III)-Citrate as electron acceptor: cell density as a function of time with lactate (A) or formate (B) as electron donor, Fe(II) concentration as a function of time with lactate (C) or formate (D) as electron donor. *S. oneidensis* MR-1 (●) and Sol mutants: d29 (○), d64 (▲), H1, (△), and B7 (■).

Sol mutants H1 and B7 displayed growth deficiencies on 2L-ferrihydrite (Figure 2.6) and Fe(III) citrate (Figure 2.7) and nearly the entire spectrum of anaerobic electron acceptors (except for B7, which retained NO_3^- , NO_2^-, and $S_2O_3^{2-}$ respiratory capability).

Chrome azurol S (CAS) was used to determine if the Sol mutants were impaired in siderophore production activity. Measurement of siderophore production activity during cell growth in aerobic liquid cultures indicated that the Sol mutants produced siderophores at wild-type rates (Figure 2.8) with the exception of H1 that produced

siderophore at 50% of the wild-type rate.

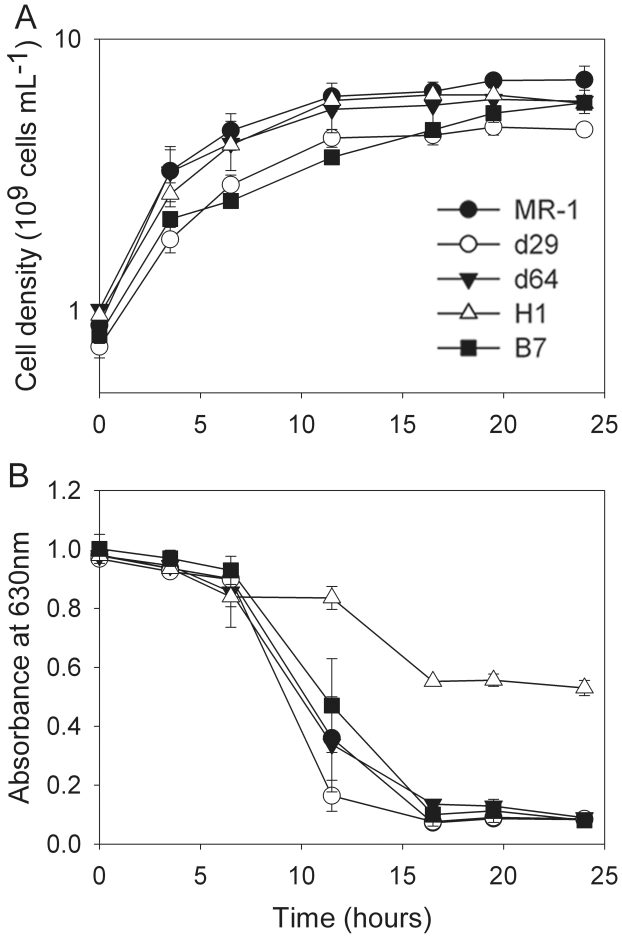

Figure 2.8 Siderophore production during growth under aerobic conditions by wild-type *S. oneidensis* (MR-1) and Sol mutants d29, d64, H1, and B7 in batch reactors with lactate as electron donor: cell density as a function of time (A) and absorbance at 630 nm of CAS-treated samples to monitor siderophore production (B). Decrease in absorbance of CAS complex at 630 nm is indicative of siderophore production. *S. oneidensis* MR-1 (●) and Sol mutants: d29 (○), d64 (▲), H1, (△), and B7 (■).

On CAS infused agar, under aerobic and anaerobic conditions, all four Sol mutants xproduced a yellow halo in the colony periphery that was similar in size to that produced by wild-type *S. oneidensis*, an indication that the Sol mutants retained siderophore production activity.

2.5 Discussion

The use of Au/Hg voltammetric microelectrodes in studies of bacterial respiratory processes is a relatively new application (Dollhopf et al., 2000; Taillefert et al., 2007). These devices have been used to follow iron and manganese reduction in batch reactors incubated with *Shewanella* species (Dollhopf et al., 2000), including the production of soluble organic-Fe(III) as an intermediate in the reduction of Fe(III) oxides (Taillefert et al., 2007). Results of the present study expand the range of *Shewanella* species capable of producing soluble organic-Fe(III) to include *S. oneidensis* MR-1. In addition, this study is the first to apply Au/Hg voltammetric microelectrodes as a screening technique to identify mutants with impaired ability to produce soluble organic-Fe(III).

Fe(III) respiratory pathways that include an initial step of non-reductive Fe(III) dissolution may provide several advantages over direct contact pathways, including raising the reduction potential of the electron acceptor to conserve more energy (Zinder et al., 1986; Taillefert et al., 2007); lowering the activation energy of the intermediate to increase reduction rates (Taillefert et al., 2007; Wang et al., 2008); binding Fe(II) products to prevent passivation of Fe(III) mineral surfaces by Fe(II) adsorption (Royer et al., 2004; Roden, 2006); or providing a bioavailable electron acceptor that may interact with periplasmic- or inner membrane-localized Fe(III) reductases (Furrer and Stumm, 1986; Duckworth and Martin, 2001). This study reveals that, soluble organic-Fe(III) is produced by *S. oneidensis* cultures incubated anaerobically with Fe(III) oxides, or with Fe(III) oxides and the alternate electron acceptor fumarate, but not in the presence of O_2, NO_3^-, or TMAO. These results suggest that soluble organic-Fe(III) production by *S. oneidensis* MR-1 may be regulated by the redox potential of terminal electron acceptors.

Soluble organic-Fe(III) is produced when grown on terminal electron acceptors with reduction potentials (E_o) around +0.05 V, the reduction potential of fumarate and 2L-ferrihydrite, but is not produced in the presence of electron acceptors with E_o values greater than +0.15 V (TMAO). Such a correlation was recently reported for *c*-type cytochrome maturation (CCM)-dependent metal respiration by *S. putrefaciens* 200 (Dale et al., 2007). A conserved histidine in cytochrome *c* maturation permease CcmB was required for anaerobic growth below a threshold E_o value of +0.36 V (NO_3^-/NO_2^- couple). Additional research will be required to determine if soluble organic-Fe(III) production by *S. oneidensis* is also regulated by E_o of the electron acceptor.

The newly developed MESA is able to measure small sample volumes (less than 100 uL) rapidly (2 hours per tray containing 96 mutagenized strains) and could be applied to multivariate analysis of biological and/or chemical reactions involving voltammetrically active species such as O_2, Fe^{2+}, Mn^{2+}, ΣH_2S, Cu^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}. Chemical mutagenesis procedures were combined with MESA to screen 3840 EMS-mutagenized strains for production of soluble organic-Fe(III). Four Sol mutants (designated d29, d64, H1, and B7) were identified with impaired ability to produce soluble organic-Fe(III) during anaerobic incubation with Fe(III) oxides (Figure 2.4).

The Fe(III)-chelating ligand has not been characterized but may originate from a variety of sources, including siderophores produced for Fe(III) assimilation, organic compounds resulting from cell lysis, or endogenous ligands specifically produced for Fe(III) respiration. While the typical binding strength of siderophores may make them inappropriate for respiratory processes, the biochemical reactions involved in Fe(III) assimilation (Wandersman and Delepelaire, 2004) are similar to those proposed for

Fe(III) solubilization and respiration (DiChristina et al., 2005). Both mechanisms include the biosynthesis of Fe(III)-chelating ligands, export of the ligands from the cell, solubilization of extracellular Fe(III) substrates, and reduction of the solublized Fe(III) via intracellular (assimilatory) or outer membrane-localized (dissimilatory) Fe(III) reductases. In the case of siderophores, however, the reductase destabilizes the Fe(III)-siderophore complex to facilitate iron removal for subsequent biochemical reactions, while the respiratory Fe(III) reductase is hypothesized to be the final step of the Fe(III)-reducing, electron transport chain (DiChristina et al., 2005; Shi et al., 2006). The siderophore production activity of three of the Sol mutants (d29, d64, and B7) is identical to wild-type *S. oneidensis* during aerobic growth (Figure 2.8), an indication that these Sol mutants are not impaired in siderophore biosynthesis. The 50% siderophore production observed by the Sol mutant H1 suggests the mutation is not in the siderophore production pathway but a general impairment to the metabolic pathway. The positive signal for siderophore production on CAS infused agar by the Sol mutants under anaerobic conditions (S2) is attributed to the removal of CAS-bound Fe(III) by the chelating ligand produced during anaerobic respiration of Fe(III) as no other electron acceptor is present in these incubations. Inadvertent cell lysis is also a possible source of Fe(III)-binding organic ligands including flavins, quinones, organic acids, organo-phosphates, and cytochromes (Hirst et al., 1999; Myers and Myers, 2004; von Canstein et al., 2008). However, soluble organic-Fe(III) is not produced by *S. oneidensis* cultures grown in the presence of 2L-ferrihydrite and the alternate electron acceptors O_2, NO_3^-, or TMAO. Soluble organic-Fe(III) complexes are also not produced by heat-killed, wild-type *S. oneidensis* cultures incubated anaerobically with 2L-ferrihydrite (Figure 2.5). These

results suggest that cell lysis products or respiration by-products are not significant sources of the voltammetric signal associated with soluble organic-Fe(III) production during anaerobic respiration of 2L-ferrihydrite.

Although the voltammetric current response should be proportional to the concentration of soluble organic-Fe(III) complexes, the exact concentration of soluble organic-Fe(III) can not be determined as unknown Fe(III)-ligand complexes can not be directly quantified by voltammetry (Taillefert et al., 2000a). The initial rate of soluble organic-Fe(III) production (21.5 nA L mg protein^{-1} hr^{-1}), however, correlates strongly (R^2 = 0.8) with the initial rate of Fe(II) production (855 mmol mg protein^{-1} hr^{-1}) by the *S. oneidensis* wild-type (24 nA soluble organic-Fe(III) per mM Fe(II)) and Sol mutant strains (Figure 2.5). This finding suggests that respiration of Fe(III) oxides proceeds via a non-reductive, Fe(III) solubilization step prior to reduction of the produced, soluble organic-Fe(III) complex. While other pathways can not be ruled out, soluble organic-Fe(III) is reduced at rates up to 20 times faster than sparingly soluble Fe(III) substrates (Arnold et al., 1988; Lovley and Woodward, 1996). If another respiration pathway were to precede the solubilization of Fe(III) oxides, the more favorable soluble Fe(III) reduction should rapidly overtake a slower pathway to become the dominant mechanism of Fe(III) reduction. The energetic cost of ligand biosynthesis, however, must be offset by greater energetic return though the Fe(III)-solubilization pathway. For instance, it has been suggested the energy spent to synthesize Fe(III)-binding ligands may be recovered by increased rates of electron transfer to Fe(III) in biofilms (Marsili et al., 2008). A group of cells working in concert to cycle ligands intercellularly may also maximize the advantages of solubilization while minimizing the biosynthetic cost of ligand production.

The four Sol mutants were tested for growth on combinations of two electron donors (lactate or formate) and nine electron acceptors (O_2, NO_3^-, NO_2^-, DMSO, $S_2O_3^{2-}$, TMAO, fumarate, soluble Fe(III)-citrate or Fe(III)-oxide). Two of the four Sol mutants (H1 and B7) display anaerobic growth deficiencies on a broad spectrum of electron acceptors (regardless of electron donor). The pleiotropic respiratory deficiencies displayed by Sol mutants H1 and B7 are identical to those displayed by other *S. oneidensis* and *S. putrefaciens* respiratory mutants previously identified for their inability to respire Fe(III), Mn(IV), U(VI), Se(IV) or Tc(VII) (DiChristina and Delong, 1994; Burnes et al., 1998; Taratus et al., 2000; Wade and DiChristina, 2000; Payne and DiChristina, 2006). Such mutants are postulated to contain mutations in regulatory components required for anaerobic gene expression (Saffarini et al., 1994). Sol mutants d29 and d64, on the other hand, are unable to respire or grow with 2L-ferrihydrite (Figure 2.6) or soluble Fe(III)-citrate (Figure 2.7), yet retain the ability to respire the remaining seven alternate electron acceptors (regardless of electron donor). The lack of growth, soluble organic-Fe(III) production, and iron reduction capability suggest that at least one component of the soluble organic-Fe(III) production system is a part of the Fe(III) respiratory pathway of *S. oneidensis* and indicate that the production of soluble organic-Fe(III) may be an important intermediate step in the anaerobic respiration of both soluble and sparingly soluble forms of Fe(III) by *S. oneidensis*. The formation of endogenous organic-Fe(III) by *S. oneidensis* when provided a soluble Fe(III) substrate suggests the endogenous ligand is not only required for solubilization of solid Fe(III) but is also required for soluble Fe(III) respiration. These results suggest that a ligand exchange reaction between endogenous and exogenous organic ligands (e.g., citrate) is required for

soluble Fe(III) respiration.

2.6 References

Arnold, R.G., Dichristina, T.J., and Hoffmann, M.R. (1988) Reductive dissolution of Fe(III) oxides by *Pseudomonas* sp. 200. *Biotechnology and Bioengineering* **32**: 1081-1096.

Brendel, P.J., and Luther, G.W. (1995) Development of a gold amalgam volammetric microelectrode for the determination of dissolved Fe, Mn, O_2 and S^{2-} in porewaters of marine and freshwater sediments. *Environmental Science & Technology* **29**: 751-761.

Bristow, G., and Taillefert, M. (2008) VOLTINT: A Matlabg (R)-based program for semi-automated processing of geochemical data acquired by voltammetry. *Computers & Geosciences* **34**: 153-162.

Burnes, B.S., Mulberry, M.J., and DiChristina, T.J. (1998) Design and application of two rapid screening techniques for isolation of Mn(IV) reduction-deficient mutants of *Shewanella putrefaciens*. *Applied and Environmental Microbiology* **64**: 2716-2720.

Carey, E., and Taillefert, M. (2005) The role of soluble Fe(III) in the cycling of iron and sulfur in coastal marine sediments. *Limnology and Oceanography* **50**: 1129-1141.

Crowe, S.A., Roberts, J.A., Weisener, C.G., and Fowle, D.A. (2007) Alteration of iron-rich lacustrine sediments by dissimilatory iron-reducing bacteria. *Geobiology* **5**: 63-73.

DiChristina, T.J., and Delong, E.F. (1994) Isolation of anaerobic respiratory mutants of *Shewanella Putrefaciens* and genetic analysis of mutants deficent in anaerobic growth on Fe^{3+}. *Journal of Bacteriology* **176**: 1468-1474.

DiChristina, T.J., Fredrickson, J.K., and Zachara, J.M. (2005) Enzymology of electron transport: Energy generation with geochemical consequences. In *Molecular Geomicrobiology*. Chantilly: Mineralogical Soc America, pp. 27-52.

Dollhopf, M.E., Nealson, K.H., Simon, D.M., and Luther, G.W. (2000) Kinetics of Fe(III) and Mn(IV) reduction by the Black Sea strain of *Shewanella putrefaciens* using in situ solid state voltammetric Au/Hg electrodes. *Marine Chemistry* **70**: 171-180.

Duckworth, O.W., and Martin, S.T. (2001) Surface complexation and dissolution of hematite by C-1-C-6 dicarboxylic acids at pH=5.0. *Geochimica Et Cosmochimica Acta* **65**: 4289-4301.

Fredrickson, J.K., and Zachara, J.M. (2008) Electron transfer at the microbe-mineral interface: a grand challenge in biogeochemistry. *Geobiology* **6**: 245-253.

Furrer, G., and Stumm, W. (1986) The coordination chemistry of weathering .1. Dissolution kinetics of delta-AL2O3 and beo. *Geochimica Et Cosmochimica Acta* **50**: 1847-1860.

Gralnick, J.A., and Newman, D.K. (2007) Extracellular respiration. *Molecular Microbiology* **65**: 1-11.

Koretsky, C.M., Moore, C.M., Lowe, K.L., Meile, C., DiChristina, T.J., and Van Cappellen, P. (2003) Seasonal oscillation of microbial iron and sulfate reduction in saltmarsh sediments (Sapelo Island, GA, USA). *Biogeochemistry* **64**: 179-203.

Kostka, J.E., Gribsholt, B., Petrie, E., Dalton, D., Skelton, H., and Kristensen, E. (2002) The rates and pathways of carbon oxidation in bioturbated saltmarsh sediments. *Limnology and Oceanography* **47**: 230-240.

Logan, B.E., Hamelers, B., Rozendal, R., Schrorder, U., Keller, J., Freguia, S. et al. (2006) Microbial fuel cells: Methodology and technology. *Environmental Science & Technology* **40**: 5181-5192.

Lovley, D.R., and Phillips, E.J.P. (1986) Organic-matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology* **51**: 683-689.

Lovley, D.R., and Phillips, E.J.P. (1988) Novel mode of microbial energy metabolism organic carbon oxidation coupled to dissimilatory reduciton of iron or manganese. *Applied and Environmental Microbiology* **54**: 1472-1480.

Lovley, D.R., and Woodward, J.C. (1996) Mechanisms for chelator stimulation of microbial Fe(III)-oxide reduction. *Chemical Geology* **132**: 19-24.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. In *Advances in Microbial Physiology, Vol. 49*. London: Academic Press Ltd, pp. 219-286.

Lovley, D.R., Baedecker, M.J., Lonergan, D.J., Cozzarelli, I.M., Phillips, E.J.P., and Siegel, D.I. (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. *Nature* **339**: 297-300.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., and Bond, D.R. (2008) *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 3968-3973.

Montgomery, H., and Dymock, J.F. (1962) The rapid determination of nitrite in fresh and saline waters. *Analyst*: 374-378.

Myers, C.R., and Nealson, K.H. (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron-acceptor. *Science* **240**: 1319-1321.

Nealson, K.H., and Saffarini, D. (1994) Iron and manganese in anaerobic respiration - Environmental significance, physiology, and regulation. *Annual Review of Microbiology* **48**: 311-343.

Nevin, K.P., and Lovley, D.R. (2002) Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. *Applied and Environmental Microbiology* **68**: 2294-2299.

Payne, A.N., and DiChristina, T.J. (2006) A rapid mutant screening technique for detection of technetium [Tc(VII)] reduction-deficient mutants of *Shewanella oneidensis* MR-1. *Fems Microbiology Letters* **259**: 282-287.

Roden, E.E. (2006) Geochemical and microbiological controls on dissimilatory iron reduction. *Comptes Rendus Geoscience* **338**: 456-467.

Royer, R.A., Dempsey, B.A., Jeon, B.H., and Burgos, W.D. (2004) Inhibition of biological reductive dissolution of hematite by ferrous iron. *Environmental Science & Technology* **38**: 187-193.

Saffarini, D.A., Dichristina, T.J., Bermudes, D., and Nealson, K.H. (1994) Anaerobic respiration of *Shewanella putrefaciens* requires both chromosomal and plasmid-brone genes. *Fems Microbiology Letters* **119**: 271-277.

Schulze-Makuch, D., Irwin, L.N., Lipps, J.H., LeMone, D., Dohm, J.M., and Fairen, A.G. (2005) Scenarios for the evolution of life on Mars. *Journal of Geophysical Research-Planets* **110**: 12.

Schwertmann, U., and Cornell, R.M. (2000) *Iron oxides in the laboratory: Preparation and characterization*. Weinheim: Wiley-VCH.

Schwyn, B., and Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47-56.

Stookey, L.L. (1970) Ferrozine - A new spectrophotometic reagent for iron. *Analytical Chemistry* **42**: 779-&.

Taillefert, M., Bono, A.B., and Luther, G.W. (2000a) Reactivity of freshly formed Fe(III) in synthetic solutions and (pore)waters: Voltammetric evidence of an aging process. *Environmental Science & Technology* **34**: 2169-2177.

Taillefert, M., Luther, G.W., and Nuzzio, D.B. (2000b) The application of electrochemical tools for in situ measurements in aquatic systems. *Electroanalysis* **12**: 401-412.

Taillefert, M., Hover, V.C., Rozan, T.F., Theberge, S.M., and Luther, G.W. (2002) The influence of sulfides on soluble organic-Fe(III) in anoxic sediment porewaters. *Estuaries* **25**: 1088-1096.

Taillefert, M., Beckler, J.S., Carey, E., Burns, J.L., Fennessey, C.M., and DiChristina, T.J. (2007) *Shewanella putrefaciens* produces an Fe(III)-solubilizing organic ligand during anaerobic respiration on insoluble Fe(III) oxides. *Journal of Inorganic Biochemistry* **101**: 1760-1767.

Taratus, E.M., Eubanks, S.G., and DiChristina, T.J. (2000) Design and application of a rapid screening technique for isolation of selenite reduction-deficient mutants of *Shewanella putrefaciens*. *Microbiological Research* **155**: 79-85.

Thamdrup, B. (2000) Bacterial manganese and iron reduction in aquatic sediments. In *Advances in Microbial Ecology, Vol 16*, pp. 41-84.

Vargas, M., Kashefi, K., Blunt-Harris, E.L., and Lovley, D.R. (1998) Microbiological evidence for Fe(III) reduction on early Earth. *Nature* **395**: 65-67.

Venkateswaran, K., Moser, D.P., Dollhopf, M.E., Lies, D.P., Saffarini, D.A., MacGregor, B.J. et al. (1999) Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *International Journal of Systematic Bacteriology* **49**: 705-724.

Wade, R., and DiChristina, T.J. (2000) Isolation of U(VI) reduction-deficient mutants of *Shewanella putrefaciens*. *Fems Microbiology Letters* **184**: 143-148.

Wandersman, C., and Delepelaire, P. (2004) Bacterial iron sources: From siderophores to hemophores. *Annual Review of Microbiology* **58**: 611-647.

Wang, Z.M., Liu, C.X., Wang, X.L., Marshall, M.J., Zachara, J.M., Rosso, K.M. et al. (2008) Kinetics of reduction of Fe(III) complexes by outer membrane cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* **74**: 6746-6755.

Watson, I.A., Oswald, S.E., Banwart, S.A., Crouch, R.S., and Thornton, S.F. (2005) Modeling the dynamics of fermentation and respiratory processes in a groundwater plume of phenolic contaminants interpreted from laboratory- to field-scale. *Environmental Science & Technology* **39**: 8829-8839.

Weber, K.A., Achenbach, L.A., and Coates, J.D. (2006) Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nature Reviews Microbiology* **4**: 752-764.

Zachara, J.M., Fredrickson, J.K., Li, S.M., Kennedy, D.W., Smith, S.C., and Gassman, P.L. (1998) Bacterial reduction of crystalline Fe^{3+} oxides in single phase suspensions and subsurface materials. *American Mineralogist* **83**: 1426-1443.

Zinder, B., Furrer, G., and Stumm, W. (1986) Coordination chemistry of weathering kinentics of the surface .2. Dissolution of Fe(III) oxides. *Geochimica Et Cosmochimica Acta* **50**: 1861-1869.

CHAPTER 3

SIDEROPHORES ARE NOT INVOLVED IN FE(III) SOLUBLIZATION DURING FE(III) RESPIRATION BY *SHEWANELLA ONEIDENSIS* MR-1

3.1 Abstract

Shewanella oneidensis MR-1 respires a wide range of anaerobic electron acceptors, including sparingly soluble Fe(III) oxides. In the present study, *S. oneidensis* was found to produce Fe(III)-solubilizing organic ligands during anaerobic Fe(III) oxide respiration, a respiratory strategy postulated to destabilize Fe(III) and produce more readily reducible, soluble organic-Fe(III). In-frame gene deletion mutagenesis, siderophore detection assays, and voltammetric techniques were combined to determine i) if the Fe(III)-solubilizing organic ligands produced by *S. oneidensis* during anaerobic Fe(III) oxide respiration were synthesized via siderophore biosynthesis systems, and ii) if the Fe(III)-siderophore reductase was required for respiration of soluble organic-Fe(III) as anaerobic electron acceptor. Genes predicted to encode the siderophore (hydroxamate) biosynthesis system (SO3030-SO3032), Fe(III)-hydroxamate receptor (SO3033), and Fe(III)-hydroxamate reductase (SO3034) were identified in the *S. oneidensis* genome, and corresponding in-frame gene deletion mutants were constructed. ΔSO3031 was unable to synthesize siderophores or produce soluble organic-Fe(III) during aerobic respiration, yet retained the ability to produce and respire soluble organic-Fe(III) (Fe(III)-citrate and Fe(III)-nitrilotriacetic acid (NTA)) at wild-type rates during anaerobic Fe(III) oxide respiration. ΔSO3034 retained the ability to synthesize siderophores during aerobic respiration and to produce and respire soluble organic-Fe(III) at wild-type rates during

anaerobic Fe(III) oxide respiration. These findings indicate that the Fe(III)-solubilizing organic ligands produced by *S. oneidensis* during anaerobic Fe(III) oxide respiration are not synthesized via the hydroxamate biosynthesis system, and that the Fe(III)-hydroxamate reductase is not essential for respiration of Fe(III)-citrate or Fe(III)-NTA as anaerobic electron acceptor.

3.2 Introduction

Bacterial electron transfer to sparingly soluble electron acceptors is a critical component of a wide variety of environmental and energy-generating processes, including biogeochemical cycling of metals, degradation of natural and contaminant organic matter, weathering of clays and minerals, biomineralization of Fe-bearing minerals, reductive precipitation of toxic metals and radionuclides, and electricity generation in microbial fuels cells (Lovley et al., 2004; DiChristina et al., 2005; Logan et al., 2006). Anaerobic and facultatively anaerobic bacteria capable of respiring sparingly soluble Fe(III) oxides are ubiquitous in nature and may be found in marine, freshwater and terrestrial environments, including metal- and radionuclide-contaminated subsurface aquifers (Lovley et al., 2004; Hau and Gralnick, 2007). Fe(III)-respiring bacteria are also deeply rooted and scattered throughout the domains *Bacteria* and *Archaea* (possibly indicating an ancient metabolic process) and include hyperthermophiles, psychrophiles, acidophiles and extreme barophiles (Lovley et al., 2004). Despite their potential environmental, energy-generating and evolutionary significance, the molecular details of microbial Fe(III) respiration remain unclear.

Fe(III)-respiring, neutrophilic bacteria are presented with a unique physiological challenge: they are required to respire anaerobically on electron acceptors found largely

as sparingly soluble Fe(III) oxides presumably unable to contact periplasmic- or inner membrane (IM)-localized electron transport systems. To overcome this problem, Fe(III)-respiring bacteria are postulated to employ novel respiratory strategies not found in other bacteria (e.g., aerobes, denitrifiers, sulfate-reducing bacteria, methanogens) that respire soluble electron acceptors (Madigan et al., 2003; DiChristina et al., 2005). The novel respiratory strategies include: 1) a direct contact pathway in which terminal Fe(III) reductases are secreted to the cell outer membrane (OM) where they contact and deliver electrons directly to external Fe(III) oxides (Myers and Myers, 1992; DiChristina et al., 2002; Reguera et al., 2005; Gorby et al., 2006; Shi et al., 2006); 2) a two-step electron shuttling pathway in which bacterially reduced endogenous or exogenous electron shuttles deliver electrons to external Fe(III) oxides in a second (abiotic) electron transfer reaction (Newman and Kolter, 2000; Coates et al., 2002; Hernandez et al., 2004; Marsili et al., 2008); and 3) a two-step Fe(III) chelation (solubilization) pathway in which Fe(III) oxides are first non-reductively dissolved by endogenous organic ligands prior to reduction of the resulting soluble organic-Fe(III) complexes (Lovley and Woodward, 1996; Taillefert et al., 2007).

The γ-proteobacterium *S. oneidensis* MR-1 can respire a variety of metals, metalloids and radionuclides as anaerobic electron acceptor, including S(0), Cr(VI), U(VI), Tc(VII), soluble organic-Fe(III) and sparingly soluble Fe(III) oxides (Myers and Nealson, 1988; DiChristina and DeLong, 1994; Wade and DiChristina, 2000; Payne and DiChristina, 2006; Burns and DiChristina, 2009). Previous studies have demonstrated that metal-respiring *S. putrefaciens* strain 200 produces Fe(III)-solubilizing organic ligands during anaerobic growth on Fe(III) oxides (Taillefert et al., 2007). In the present

study, *S. oneidensis* MR-1 is also found to produce Fe(III)-solubilizing organic ligands during anaerobic Fe(III) oxide respiration. The Fe(III)-solubilizing organic ligands are postulated to facilitate anaerobic Fe(III) respiration by destabilizing Fe(III) from the surface of Fe(III) oxide substrates. Soluble organic-Fe(III) production may function as a requisite intermediate step in anaerobic respiration of sparingly soluble Fe(III) oxides (Taillefert et al., 2007). The Fe(III)-solubilizing organic ligands produced by *S. oneidensis* during anaerobic Fe(III) oxide respiration, however, have yet to be identified.

Candidate organic ligands for production of soluble organic-Fe(III) during anaerobic Fe(III) oxide respiration include siderophores, the Fe(III)-chelating compounds synthesized and secreted by a wide variety of bacteria and fungi for solubilization and subsequent assimilation of otherwise inaccessible Fe(III) substrates (Crosa, 1989; Neilands, 1995; Renshaw et al., 2002; Vraspir and Butler, 2009). Siderophores are categorized into three major classes according to the chemical structure of their Fe(III)-binding motifs: catecholates, α-hydroxycarboxylates, and hydroxamates. Siderophores are generally synthesized by non-ribosomal peptide synthase systems that produce thioester-linked, amino acid derivatives capable of non-reductive solubilization of amorphous (e.g., hydrous Fe(III) oxide) or crystalline (e.g., hematite, goethite) Fe(III) oxides (Hersman et al., 1995; Holmen and Casey, 1996). Hydroxamate-type siderophores are produced from L-ornithine (with glutamate as starting substrate), via N^6-hydroxylation, N^6-acylation and, in some cases, cyclization to macrocyclic ring structures (Crosa and Walsh, 2002). The macrocyclic siderophores bisucaberin and putrebactin (dimer of succinyl-(*N*-hydroxyputrescine)), for example, are two structural analogs of the cyclic bis(hydroxamate) siderophore alcaligin synthesized by *Aliivibrio salmonicida* and

S. putrefaciens strain 200, respectively (Ledyard and Butler, 1997; Winkelmann et al., 2002; Hjerde et al., 2008) .

After recognition by Fe(III)-siderophore-specific receptors located on the cell surface, the Fe(III)-siderophore complexes are transported across the cell envelope by energy (TonB)-dependent pathways (Moeck and Coulton, 1998). Fe(III) is subsequently released from the Fe(III)-siderophore complex by ligand exchange reactions promoted by siderophore ligand hydrolysis and/or protonation or by Fe(III)-siderophore reduction and release of Fe(II) to acceptor ligands (Caldwell and Crumbliss, 1998; Wirgau et al., 2002). Fe(III)-siderophore reductases generally contain co-factors such as flavins or Fe-S clusters that catalyze electron transfer to siderophore-bound Fe(III) (Schroder et al., 2003). The Fe-S protein FhuF of *Escherichia coli*, for example, catalyzes the reductive release of Fe(II) from Fe(III)-hydroxamate complexes (Matzanke et al., 2004).

The main objectives of the present study were to determine i) if the Fe(III)-solubilizing organic ligands produced by *S. oneidensis* during anaerobic Fe(III) oxide respiration are synthesized by Fe(III)-siderophore biosynthesis systems, and ii) if Fe(III)-siderophore reductases are required for respiration of soluble organic-Fe(III) as anaerobic electron acceptor. The experimental strategy for this study included 1) identification of genes encoding the siderophore biosynthesis and Fe(III)-siderophore reductase systems in the *S. oneidensis* genome, 2) generation of in-frame deletions in the corresponding siderophore biosynthesis and Fe(III)-siderophore reductase genes, 3) tests of the resulting siderophore biosynthesis mutants for production of siderophores and soluble organic-Fe(III) during aerobic and anaerobic Fe(III) oxide respiration, and 4) tests of the resulting

Fe(III)-siderophore reductase mutants for respiration of soluble organic-Fe(III) as anaerobic electron acceptor.

3.3 Materials and Methods

Growth media and cultivation conditions.

All bacterial strains and plasmids used in this study are listed in Table 3.1.

Table 3.1. Strains and plasmids used in this study.

Strain	Features	Source
Shewanella oneidensis		
MR-1	Wild-type strain	ATCC
ΔSO3031	in-frame deletion mutant	This study
ΔSO3034	in-frame deletion mutant	This study
ΔSO3031-KI	"knock-in" complemented mutant	This study
ΔSO3034-KI	"knock-in" complemented mutant	This study
Escherichia coli		
EC100D *pir*-116	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74recA1 endA1 araD139 Δ(ara,leu)7697 galU galK λ- rpsL nupG pir-116(DHFR)	Epicentre
β 2155 λ pir	*thrB1*004*pro thi strA hsdS lacZ*Δ*M15* (F9 *lacZ*Δ*M15 lacI*q *traD36 proA*1 *proB*1 Δ*dapA::erm pir::*RP4 Km^R	Dehio and Meyer 1997
Plasmids		
pKO2.0	4.5 kb γR6K, *mob*RP4 *sacB* Gm^R *lacZ*	Burns and DiChristina 2009
pKOSO3031	pKO2.0 containing in-frame deletion of SO3031	This study
pKOSO3034	pKO2.0 containing in-frame deletion of SO3034	This study
pKOSO3031KI	pKO2.0 containing WT SO3031	This study
pKOSO3034KI	pKO2.0 containing WT SO3034	This study

For genetic manipulations, *S. oneidensis* MR-1 was cultured at 30°C in Luria-Bertani (LB) medium (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone). For anaerobic growth experiments, cells were cultured in a defined salts medium (SM) (DiChristina and

DeLong, 1994) supplemented with lactate (18 mM) or formate (30 mM) as carbon/energy source under a nitrogen atmosphere. When required, antibiotics were supplemented at the following final concentrations: gentamycin, 15 μg mL^{-1} and chloramphenicol, 25 μg mL^{-1}. For growth of *Escherichia coli* β2155 λ pir (12), diaminopimelate (DAP) was supplemented at a final concentration of 100 μg mL^{-1}. 2,2'-dipyridyl experiments were carried out in SM liquid medium amended with 2,2'-dipyridyl at a range of concentrations (0-200 μM) under aerobic growth conditions. Aerobic growth was monitored spectrophotometrically by measuring changes in absorbance at 600 nm.

Nucleotide and amino acid sequence analyses.

S. oneidensis genome sequence information was obtained from the Comprehensive Microbial Resource (J. Craig Venter Institute, http://cmr.jcvi.org). Proteins displaying sequence similarity to SO3030-SO3034 were identified via blastp analysis available at the National Center for Biotechnology Information (NCBI)(Altschul et al., 1997). Conserved protein domains were identified with Pfam (http://pfam.sanger.ac.uk)(Finn et al., 2008) and Prosite (http://www.expasy.org/prosite/) (Hulo et al., 2006) software. Multiple sequence alignments were performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

In-frame gene deletion mutagenesis.

Genes encoding SO3031 and SO3034 were deleted in-frame from the *S. oneidensis* genome as described previously (Burns and DiChristina, 2009). Regions corresponding to ~750 bp upstream and downstream of each open reading frame were PCR-amplified with iProof ultrahigh-fidelity polymerase (Bio-Rad, Hercules CA)

generating fragments F1 and F2 which were fused by overlap extension PCR to generate fragment F3. Primers for construction of ΔSO3031 and ΔSO3034 are listed in Table 3.2.

Table 3.2. Primers used in this study

Primer	Sequence
SO3031D1	GACTGGATCCCACTAAACTGACCCTAGAGATGACCTCG
SO3031D2	AAAGTGACTGGGTAACAGGGCGATTATCTGCACAACGTCTGCC CATTG
SO3031D3	CAATGGAGCAGACGTTGTGCAGATAATCGCCCTGTTACCCAGTC ACTTT
SO3031D4	GACTGTCGACCGCACTGCAATCCACACGAGTTTT
SO3031DTF	GCCGCAAATTGGTATTAGGAACAGGC
SO3031DTR	GCTCTCGCCAAG GTAAATAATGTCGC
SO3034D1	GACTGGATCCAAGACAATGTTGGCGAACGCACTG
SO3034D2	CTAATGGGCAATCACCGCATTGATGTTGGATGAGGGTTGGAAT CGCTTC
SO3034D3	GAAGCGATTCCAACCCTCATCCAACATCAATGCGGTGATTGCCC ATTAG
SO3034D4	GACTGTCGACTGATATACACCTGCGGCGAAGTAAGG
SO3034TF	GCAACTTAATCTGACCGATGCCTTGG
SO3034TR	CGTGGTAATGTACGTGATAGCCCATG
3031C1	GACTCTCGAGCACTAAACTGACCCTAGAGATGACCTCG
3031C4	GACTCCGCGGCGCACTGCAATCCACACGAGTTTT

Fragment F3 was cloned into pKO2.0 with BamH1 and SalI restriction endonucleases and electroporated into *E. coli* strain β2155 λ pir. pKO2.0-F3 was mobilized into recipient *S. oneidensis* MR-1 via biparental mating procedures (Dehio and Meyer, 1997). A plasmid integrant was identified via PCR analysis, and the mutation was resolved on LB agar containing sucrose (10%). Transconjugates were identified on SM agar containing Gm and confirmed via PCR. Following counterselection on SM agar containing sucrose (10% w/v), the corresponding in-frame deletion mutant strains

(designated ΔSO3031 and ΔSO3034, respectively) were isolated and confirmed via PCR and DNA sequencing (University of Nevada, Reno Genomics Facility).

Knock-in complementation analysis.

ΔSO3031 and ΔSO3034 were each complemented via a knock-in complementation strategy similar to that followed in the in-frame gene deletion protocol described above. Wild-type copies of SO3031 and SO3034 were PCR-amplified from wild-type *S. oneidensis* genomic DNA with SO3031- and SO3034-specific primers 3031C1 and 3031C2 and SO3034D1 and SO3034D4, respectively (Table 3.2). The resulting amplicon contained the entire ORF and ~750 bp of upstream and downstream DNA for subsequent recombination into ΔSO3031 and ΔSO3034. The amplicons were cloned into pKO2.0 using identical restriction sites and the resulting constructs were subsequently transformed into *E. coli* strains as described above. Knock-in complementation was performed as described for in-frame deletion above, with the exception that ΔSO3031 and ΔSO3034 were used as recipient strains to generate the corresponding knock-in complemented strains ΔSO3031-KI and ΔSO3034-KI, respectively. Insertion into the proper location of the ΔSO3031-KI and ΔSO3034-KI genomes was confirmed via PCR amplification with flanking primers SO3031DTF and SO3031DTR for ΔSO3031 and SO3034DTF and SO3034DTR for ΔSO3034 (Table 3.2) followed by DNA sequencing (University of Nevada, Reno Genomics Center).

Determination of overall respiratory capability of *S. oneidensis* wild-type, ΔSO3031, and ΔSO3034 mutant strains.

S. oneidensis wild-type, ΔSO3031, and ΔSO3034 mutant strains were inoculated in SM liquid growth medium (initial concentration of 10^7 cells mL^{-1}) amended with either

18 mM lactate or 30 mM formate as electron donor and either O_2, 15 mM nitrate, 50 mM dimethylsulfoxide (DMSO), 25 mM trimethylamine-*N*-oxide (TMAO), 10 mM fumarate, 10 mM thiosulfate, 50 mM Fe(III)-citrate, colloidal 10 mM MnO_2, 10 mM Mn(III)-pyrophosphate, 20 mM Fe(III)-desferrioxamine B (DEFB), 20 mM Fe(III)-NTA, or 40 mM hydrous Fe(III)-oxide (HFO) as electron acceptor. For aerobic growth, compressed air was vigorously bubbled through the reactors. Anaerobic conditions were maintained by continuous sparging with N_2(g). Cell growth was monitored by measuring absorbance at 600 nm over time. Nitrite (NO_2^-) was measured by diluting samples 250-fold in a solution consisting of 9.6 mM sulfanilic acid, 96 mM $KHSO_4$, and 3.2 mM N,N-ethylenediamine (Montgomery and Dymock, 1962). Samples were held in the dark for 15 minutes prior to absorbance measurements at 510 nm. Mn(III) reduction was monitored colorimetrically by filtering samples through a disposable nylon filter with a pore size of 0.22 μm (millex) and subsequently monitoring absorbance spectrophotometrically at 480 nm (Kostka et al., 1995). Mn(IV) reduction was monitored by mixing samples in a 1:9 ratio with a 2mM benzidine in 10% acetic acid solution and monitoring absorbance spectrophotometrically at 424nm (Burnes et al., 1998; Dale et al., 2007), Fe(III) reduction rates were determined by monitoring Fe(II) production over time via HCl extraction followed by the Ferrozine colorimetric assay (Stookey, 1970; Lovley and Phillips, 1986). Cell growth was monitored by direct cell counts of acridine orange-stained cells via epifluorescence microscopy (Carl Zeiss AxioImager Z1 Microscope) (Dale et al., 2007; Burns and DiChristina, 2009).

Siderophore detection via chrome azurol-S (CAS)-based techniques.

Siderophores were detected during growth on liquid or solid SM growth media

via application of CAS-based techniques. CAS screening plates were prepared using a modified version of a previously described procedure (Schwyn and Neilands, 1987). Blue CAS (Sigma Aldrich) agar was prepared by adding 60.5 mg of CAS dye dissolved in 50 mL water to 10mL acidic solution of $FeCl_3$ (1 mM $FeCl_3$, 10 mM HCl). This mixture was slowly added to 40 ml of a 0.2 mM solution of hexadecyltrimethylammonium (HDTMA) (Sigma Aldrich), and the resulting solution was autoclaved, cooled to 55ºC and added to 900 mL sterile SM growth medium supplemented with 1.5% w/v agar (CAS agar). CAS shuttling solution was prepared as previously described (Schwyn and Neilands, 1987). Siderophore production by *S. oneidensis* wild-type and ΔSO3031 and ΔSO3034 mutant strains was monitored by patching colonies onto CAS agar plates, incubating aerobically for 24 hours and visually scoring the colony periphery for yellow halos. Siderophore production was also monitored during aerobic growth in liquid SM medium with lactate as electron donor. Samples were centrifuged for 1 min (12,000x*g*), and the resulting supernatant was mixed with 0.5 mL CAS shuttling solution and incubated for 3 hours. Samples were subsequently measured spectrophotometrically at 630 nm to determine siderophore concentration.

Under anaerobic Fe(III) oxide-respiring growth conditions, a modified CAS assay was employed to detect production of Fe(III)-chelating organic ligands. In the modified CAS assay, aliquots from anaerobic Fe(III) oxide-respiring cultures were withdrawn and centrifuged at 12,000x*g* for 1 min, and the resulting supernatant reacted with Fe(III)-free CAS shuttling solution for 6 hours prior to measuring Fe(III)-CAS complex formation colorimetrically at 630 nm. The modified CAS assay facilitates detection of organic

ligands with Fe(III) binding affinities that are lower than those observed with traditional siderophores (the traditional CAS assay detects a ligand exchange reaction between Fe(III)-CAS and a competing siderophore). Production of catecholate-type siderophores was determined with the Arnow assay by reacting culture supernatants with equal volumes of 0.5 M HCl, a solution of 10% w/v sodium nitrate and 10% w/v sodium molybdate, and 1.0 M NaOH. The presence of catecholate ligands was determined spectrophotometrically at 505 nm (Arnow, 1937).

Detection of soluble organic-Fe(III) via voltammetric analyses.

S. oneidensis wild-type, ΔSO3031, and ΔSO3034 mutant strains were incubated in 100 mL PEEK™ batch reactors containing SM liquid growth medium supplemented with 30 mM HFO as electron acceptor and 20 mM sodium lactate as electron donor. Reactors were inoculated with cells at an initial concentration of 2 x 10^7 cells mL^{-1} and incubated in a Coy chamber under anaerobic conditions (atmosphere of 85% N_2, 10% CO_2, 5% H_2) with gentle mixing. An aliquot from each vessel was extracted in 0.5 M HCl for total Fe(II) measurement via the Ferrozine colorimetric method (Stookey, 1970; Lovley and Phillips, 1986). Soluble organic Fe(III) was measured using voltammetric microelectrodes and a computer-operated DLK-100A potentiostat (Analytical Instrument Systems, Inc.) (Taillefert et al., 2007). Electrodes for voltammetric analysis consisted of 100 μm diameter gold-mercury (Au/Hg) amalgam working electrodes and 0.5 mm diameter Ag/AgCl reference and platinum counter electrodes. Working electrodes were polished, plated and conditioned as previously described (Brendel and Luther, 1995). Voltammetric conditions used in triplicate cathodic square wave voltammetry (CSWV) measurements included: preconditioning of -0.9 V for 10 sec and deposition at -0.1 V for

10 sec to concentrate Fe(III) species at the electrode surface. A scan rate of 200 mV s^{-1} from -0.1 to -1.8 V with a pulse height of 0.05 V was used as previously described (Taillefert et al., 2000; Carey and Taillefert, 2005; Taillefert et al., 2007; Tercier-Waeber and Taillefert, 2008). Electrodes were calibrated for Mn^{2+} (MDL ~ 5 μmol L-1) by cathodic square wave voltammetry (CSWV) in degassed medium and the pilot ion method was used to quantify concentrations of Fe(II). Voltammograms were integrated using a semi-automated software developed for these applications (Bristow and Taillefert, 2008). Initial rates of soluble organic-Fe(III) production were determined by linear regression of the increase in soluble organic-Fe(III) current intensities for 24 hours (disregarding any initial lag period). Initial rates of Fe(III) reduction were determined by linear regression of the total Fe(II) production rate during the period of soluble organic-Fe(III) production. All rates were normalized to cellular protein content determined via the Bradford assay (Bradford, 1976).

3.4 Results

Genome-wide sequence analysis identified an *S. oneidensis* gene cluster (SO3030-SO3034) whose predicted proteins displayed high homology to enzymes encoding the hydroxamate synthesis system, Fe(III)-hydroxamate receptor and Fe(III)-hydroxamate reductase of other gram-negative γ-proteobacteria (Table 3.3).

Table 3.3. Identities and similarities between SO3030-SO3034 homologs and the homolog of highest similarity outside the genus *Shewanella* (best-hit) in GenBank and putative function.

ORF	*Shewanella* spp.[a]			GenBank [b]				Putative function
	Sim	ID	E value	Best hit	Sim	ID	E value	
SO3030	52-89	29-83	10^{-57} - 0.0	*Aliivibrio salmonicida* LFI1237	72	56	10^{-149}	lysine-N6 hydroxylase
SO3031	74-89	63-77	10^{-71} - 10^{-87}	*Aliivibrio salmonicida* LFI1238	64	46	10^{-41}	acyl-CoA N-acyl transferase
SO3032	38-94	22-89	10^{-7} - 0.0	*Aliivibrio salmonicida* LFI1239	75	59	0.0	non-ribsomal peptide synthase
SO3033	40-98	22-96	10^{-14} - 0.0	*Aliivibrio salmonicida* LFI1240	72	53	0.0	tonB-dependant hydroxamate-type ferrisiderophore receptor
SO3034	47-65	39-53	10^{-57} - 10^{-98}	*Enterobacter sp. 638*	41	28	10^{-20}	ferric iron reductase involved in ferric hydroxamate transfer

[a] Percent sequence similarity (Sim), identity (ID) and expect value (E value) between *S. oneidensis* SO3030-SO3034 sequences obtained from TIGR. Ranges were determined by pairwise comparison with translated sequence data from genome sequences of recently sequenced *Shewanella*, including *S. putrefaciens* 200, *S. putrefaciens* CN32, *S. putrefaciens* W3-18-1, *S. amazonensis* SB2B, *S. denitrificans* OS217, *S baltica* OS195, *S. frigidimarina* NCIMB400, *S. pealeana* ATCC 700345, *S. woody* ATCC 51908, *S.* sp. ANA-3, *S.* sp. MR-4, *S.* sp. MR-7, *S. loihica* PV-4, *S. halifaxens*, *S. piezotolerans*, *S. benthica*, and *S. sediminis*.
[b] Organisms outside the genus *Shewanella* with the homolog of highest similarity (Best hit) determined by BlastP analysis of GenBank non-redundant database.

Genes encoding synthesis systems for production of catecholate- and α-hydroxycarboxylate-type siderophores were not detected (data not shown). *S. oneidensis* proteins SO3030-SO3033 displayed high similarity (64-75%) and correspondingly low e-values (0.0-10^{-41}) to the lysine-N_6-hydroxylase (SO3030), acyl-CoA-N-acyl-transferase (SO3031), non-ribsomal peptide synthase (SO3032), and tonB-dependent, hydroxamate-type Fe(III)-siderophore receptor (SO3033) found in the LF11237-LF11240 gene cluster of *A. salmonicida* (Table 3.3). *S. oneidensis* SO3034 displayed moderate similarity (41%) and e-value (10^{-20}) to the *E. coli* FhuF-like, Fe(III)-hydroxamate reductase of *Enterobacter sp.* 638 (Table 3.3).

The SO3030-SO3034 gene cluster of *S. oneidensis* also displayed high similarity (75-93%) and corresponding e-values ($0.0\text{-}10^{-72}$) to a gene cluster identified in the unannotated genome of *S. putrefaciens* strain 200, a phylogenetically related strain known to produce the macrocyclic (dihydroxamate-type) siderophore putrebactin (Ledyard and Butler, 1997), and to produce soluble organic-Fe(III) during anaerobic Fe(III) oxide respiration (Taillefert et al., 2007). Additional genome-wide sequence analyses indicated that proteins with moderate-to-high similarity to the *S. oneidensis* SO3030-SO3034 gene cluster were present in all recently sequenced *Shewanella* genomes, including *S. putrefaciens* CN32, *S. putrefaciens* W3-18-1, *S. amazonensis* SB2B, *S. denitrificans* OS217, *S. baltica* OS195, *S. frigidimarina* NCIMB400, *S. pealeana* ATCC 700345, *S. woodyi* ATCC 51908, *S.* sp. ANA-3, *S.* sp. MR-4, *S.* sp. MR-7, *S. loihica* PV-4, *S. halifaxens*, *S. piezotolerans*, *S. benthica*, and *S. sediminis* (Table 3.3).

The genes encoding SO3031 and SO3034 were deleted in-frame, and the resulting mutants (ΔSO3031 and ΔSO3034, respectively) were tested for aerobic and anaerobic growth on a suite of 11 electron acceptors. ΔSO3031 and ΔSO3034 retained the ability to grow at wild-type rates on all electron acceptors (with lactate or formate as electron donor) including sparingly soluble Fe(III) oxides (HFO) and soluble organic-Fe(III) (supplied as Fe(III)-citrate and Fe(III)-NTA) (Figures. 3.1 and 3.2).

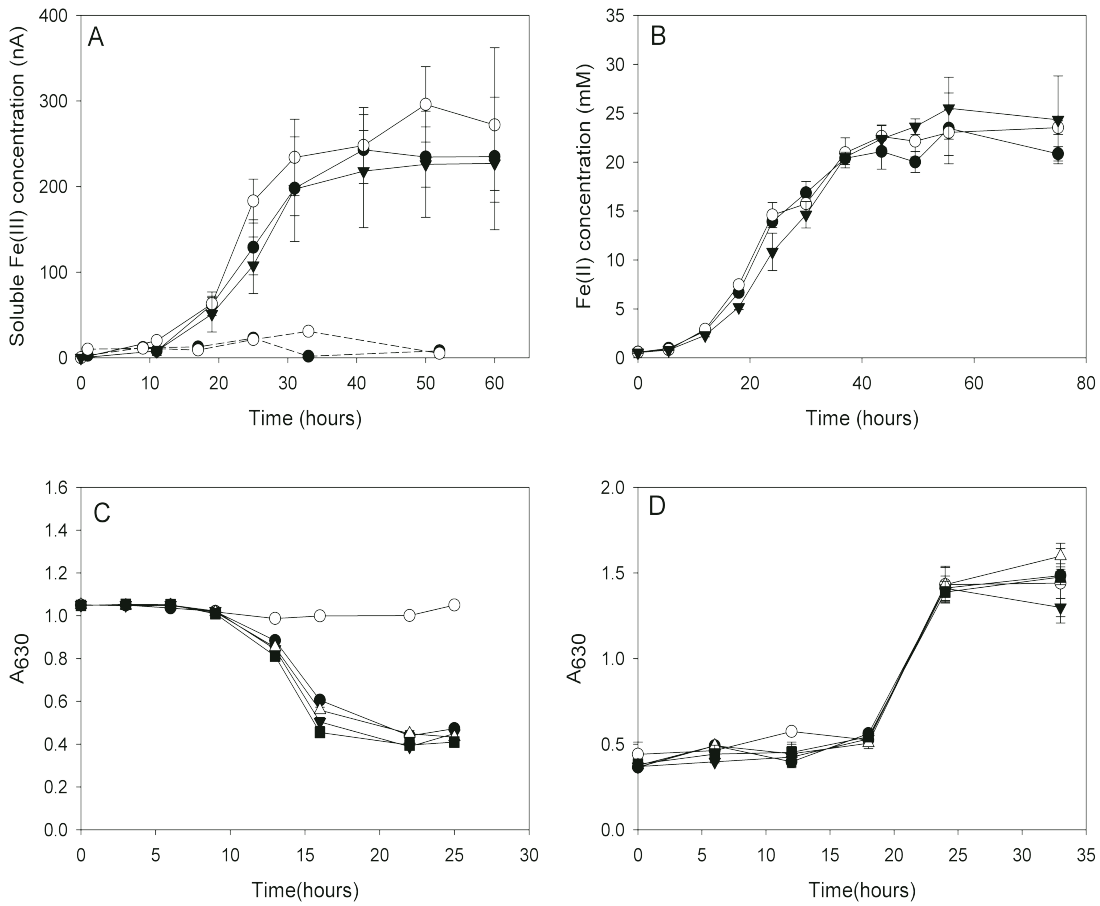

Figure 3.1 Siderophore production and Fe(III) reduction. Wild-type (●), ΔSO3031(○), ΔSO3034 (▼), ΔSO3031-KI (△), and ΔSO3034-KI (■). A) Soluble Fe(III) production as determined via square-wave voltammetry anaerobically (—) and aerobically (---) in incubations with HFO and lactate as electron donor. B) Fe(II) concentration in incubations of HFO as electron acceptor and lactate as electron donor. C) Absorbance at 630 nm of CAS treated samples monitoring siderophore production in incubations with O_2 as electron acceptor and lactate as electron donor. D) Absorbance at 630 nm of iron-free-CAS treated samples monitoring soluble Fe(III) production with HFO as electron acceptor and lactate as electron donor. Points plotted represent the mean of duplicate measurements carried out in two parallel, yet independent incubations. In some cases, error bars are smaller than symbol.

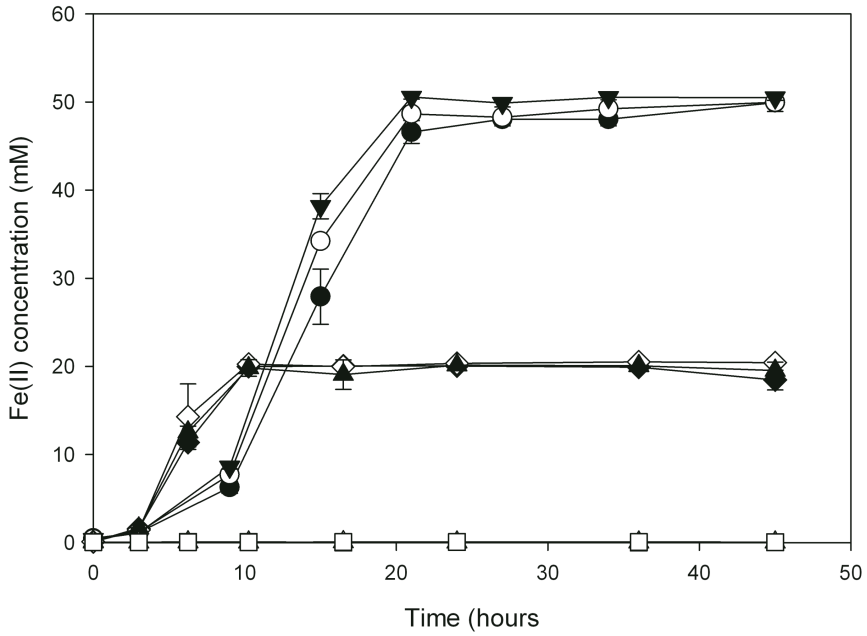

Figure 3.2 Soluble organic-Fe(III) reduction. Fe(II) concentration in incubations of 50 mM Fe(III)-citrate (Wild-type (●), ΔSO3031(○), ΔSO3034 (▼)), 20 mM Fe(III)-NTA (Wild-type (◆), ΔSO3031(◇), ΔSO3034(▲)) or 20 mM Fe(III)-DEFB (Wild-type (△), ΔSO3031(■), ΔSO3034(□) as electron acceptor and lactate as electron donor. Points plotted represent the mean of duplicate measurements carried out in two parallel, yet independent incubations. In some cases, error bars are smaller than symbol.

Wild-type *S. oneidensis* was unable to respire anaerobically on soluble organic-Fe(III) supplied as Fe(III)-DEFB (Figure 3.2). To differentiate the aerobic growth deficiency of ΔSO3031 from wild-type *S. oneidensis,* the Fe(II)-scavenging compound 2,2'-dipyridyl was added to SM medium prior to inoculation. At 2,2'-dipyridyl concentrations >200 μM, neither ΔSO3031, ΔSO3034, nor the wild-type strain were capable of aerobic growth (data not shown), an indication that Fe was scavenged below threshold Fe levels required for aerobic growth of all strains, including the wild-type. While ΔSO3031 was unable to grow aerobically in the presence of 2,2'-dipyridyl concentrations of 100 μM, the wild-

type, ΔSO3034, ΔSO3031-KI, and ΔSO3034-KI strains grew aerobically at normal rates (Figure 3.3).

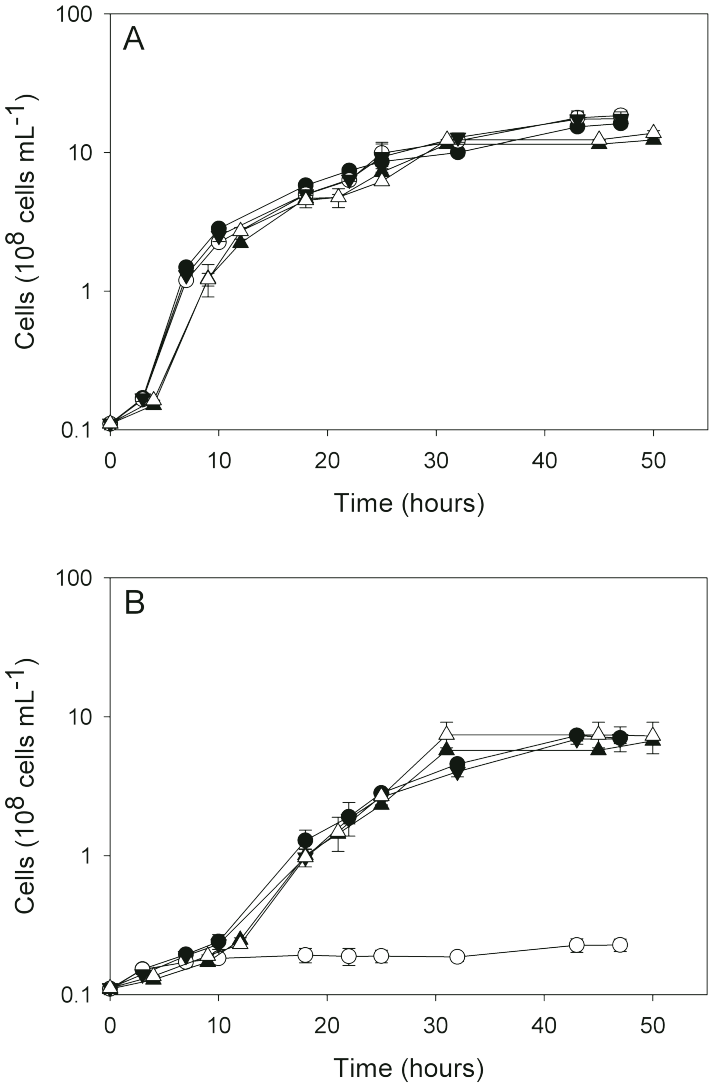

Figure 3.3 Mutant growth capabilities in the presence of 2,2' dipyridyl. Wild-type (●), ΔSO3031(○), ΔSO3034 (▼), ΔSO3031-KI (△), and ΔSO3034-KI (▲). A) Cell density as a function of time with O_2 as electron acceptor and lactate as electron donor in the presence of 0 μM 2,2' dipyridyl. B) Cell density as a function of time with O_2 as electron acceptor and lactate as electron donor in the presence of 100 μM 2,2' dipyridyl. Points plotted represent the mean of duplicate measurements carried out in two parallel, yet independent incubations. In some cases, error bars are smaller than symbol.

Under aerobic growth conditions (and in the absence of 2,2'-dipyridyl), ΔSO3034, ΔSO3031-KI and ΔSO3034-KI strains produced CAS-reactive siderophores at wild-type rates (Figure 3.1). ΔSO3031, on the other hand, was unable to produce siderophores under aerobic growth conditions (in the absence of 2,2'-dipyridyl). Aerobic incubations on CAS-supplemented agar plates demonstrated that all strains except ΔSO3031 produced a yellow halo around the colony periphery, an indication that only ΔSO3031 lacked the ability to produce a diffusible, Fe(III)-chelating compound (presumably hydroxamate) that outcompeted CAS for Fe(III) (Figure 3.4).

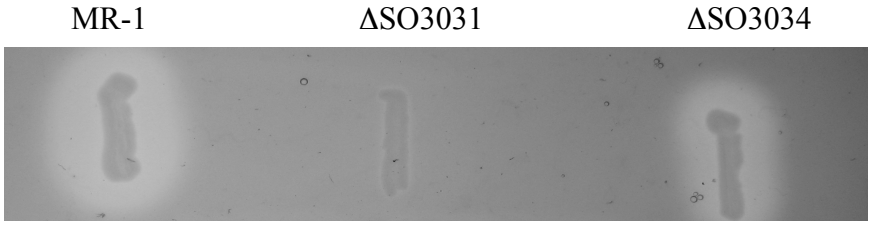

Figure 3.4 Determination of siderophore production via the CAS assay. Wild-type, ΔSO3031, ΔSO3034 (left to right) colonies patched onto SM-CAS agar plates and grown aerobically for 24 hrs. Clearing zones around patch periphery indicates production of Fe(III)-chelating siderophores.

Arnow-based assays revealed that none of the strains (including the wild-type) produced catecholate-type siderophores at detectable levels during aerobic growth (in the absence of 2,2'-dipyridyl)(data not shown). Under anaerobic Fe(III)-oxide respiring growth conditions, all strains (including ΔSO3031) produced CAS-reactive Fe(III)-chelating ligands at wild-type rates (detected via the modified CAS assay, Figure 3.1).

Voltammetric analyses indicated that both ΔSO3031 and ΔSO3034 strains produced soluble organic-Fe(III) complexes (at rates corresponding to 0.03 nA mg

protein^{-1} h^{-1}) and reduced Fe(III) oxides at wild-type rates (500 μmol mg protein^{-1} hr^{-1}**)** during anaerobic growth on Fe(III) oxides as electron acceptor (Figure 3.1). *S. oneidensis* wild-type, ΔSO3031, and ΔSO3034 strains, on the other hand, produced soluble organic-Fe(III) at or below detection limits during aerobic growth in the presence of Fe(III) oxides (Figure 3.1). Neither soluble organic-Fe(III) nor Fe(II) were detected in abiotic or heat-killed controls carried out under aerobic or anaerobic conditions (data not shown).

3.5 Discussion

Previous voltammetric analyses indicated that *S. putrefaciens* strain 200 produced soluble organic-Fe(III) during anaerobic respiration of sparingly soluble Fe(III) oxides, including hematite, goethite, and 2-line ferrihydrite (Taillefert et al., 2007). Results of the present study expand the range of *Shewanella* species capable of producing soluble organic-Fe(III) during anaerobic Fe(III) oxide respiration to include *S. oneidensis*. Soluble organic-Fe(III) production may therefore be a requisite intermediate step in anaerobic respiration of Fe(III) oxides by metal-respiring members of the *Shewanella* genus. Previous studies indicated that a main pathway for Fe(III) oxide respiration by *S. oneidensis* entailed direct contact between Fe(III) oxides and cell surface-exposed *c*-type cytochromes such as MtrC and OmcA (Xiong et al., 2006; Shi et al., 2007). Subsequent electrochemical analyses revealed that a distance of <15 Å was required for electron transfer from the catalytic heme group of OmcA to hematite (Kerisit et al., 2007). The direct contact mechanism was recently brought into question, however, by kinetic analyses which demonstrated that OmcA and MtrC are kinetically competent to account for whole cell reduction of soluble-organic Fe(III), but not for sparingly soluble Fe(III)

oxides (Ross et al., 2009). Fe(III) oxide respiratory pathways that include an intermediate step of non-reductive Fe(III) dissolution may provide several distinct advantages over the direct contact pathway. Soluble organic-Fe(III) produced during non-reductive Fe(III) dissolution may display i) higher reduction potentials to conserve more energy (Zinder et al., 1986; Taillefert et al., 2007), ii) lower activation energies to increase reduction rates (Taillefert et al., 2007; Marshall et al., 2008), iii) higher Fe(II)-binding affinities to prevent Fe(II) passivation of Fe(III) oxide surfaces (Royer et al., 2004; Roden, 2006), or iv) higher solubility to facilitate uptake and reduction by periplasmic- or IM-localized terminal Fe(III) reductases (Duckworth and Martin, 2001; Ruebush et al., 2006; Ross et al., 2007).

Soluble organic-Fe(III) production by Fe(III) oxide-respiring *S. putrefaciens* strain 200 was hypothesized to entail Fe(III) destabilization by bacterially produced organic ligands with high Fe(III)-chelating capability (Taillefert et al., 2007). Siderophores constitute a major class of bacterially-produced compounds with high Fe(III)-chelating capability (conditional stability constants ranging from log K=10 to 60) (Kalinowski et al., 2000; Vraspir and Butler, 2009). Several lines of evidence indicate that *S. oneidensis* synthesizes hydroxamate-like siderophores. Similar to the structurally analogous siderophore bisucaberin (log K=32) synthesized by *A. salmonicida* (Winkelmann et al., 2002), the macrocyclic (dihydroxamate) siderophore putrebactin is synthesized by *S. putrefaciens* strain 200 (Ledyard and Butler, 1997). Correspondingly, a contiguous five-gene cluster predicted to encode an *A. salmonicida*-like hydroxamate synthesis system (SO3030-3032), Fe(III)-hydroxamate receptor (SO3033), and Fe(III)-hydroxamate reductase (SO3034) was identified in the genomes of both *S. oneidensis* and

S. putrefaciens strain 200 (which displayed nearly identical sequence similarity). Genes encoding catecholate- or α-hydroxycarboxylate-type siderophores were not detected in either genome, an indication that siderophore production by both *Shewanella* strains was based on the hydroxamate synthesis system.

Results of CAS-based siderophore detection assays confirmed that wild-type *S. oneidensis* produces siderophores during aerobic growth, while ΔSO3031 (harboring an in-frame deletion of the predicted acyl-coA-N^6-acyl transferase) was unable to produce siderophores during aerobic growth. *S. oneidensis* wild-type, ΔSO3031, and ΔSO3034 mutant strains retained the ability to grow aerobically or anaerobically on a suite of 11 electron acceptors without addition of exogenous Fe for assimilatory purposes. Aerobic growth of ΔSO3031 was impaired only if the Fe(II)-scavenging compound 2,2'-dipyridyl was supplied at 100 μM levels (aerobic growth of wild-type and ΔSO3034 strains were not affected at this level). Background Fe levels in SM Medium (below detection limits 0.1 μM Fe(II)) were evidently high enough to sustain aerobic growth of ΔSO3031 even in the absence of the Fe(III)-hydroxamate biosynthesis system. Such Fe-scavenging capability was also recently reported in a study of the ferric uptake regulator (Fur) regulon of *S. oneidensis* (Yang et al., 2008). In that study, addition of 2,2'-dipyridyl was also required to differentiate aerobic growth of *S. oneidensis* wild-type and *fur* mutant strains. In contrast to that observed under aerobic growth conditions, voltammetric and CAS-based analyses demonstrated that ΔSO3031 produced soluble organic-Fe(III) under anaerobic Fe(III) oxide-respiring conditions. Since the hydroxamate biosynthesis synthesis system was disabled in ΔSO3031, this finding indicates that the Fe(III)-chelating ligands produced for anaerobic Fe(III) respiration were not synthesized by the

hydroxamate system. Genes encoding other siderophore synthesis systems were not detected in the *S. oneidensis* genome, an indication that the Fe(III)-chelating ligands produced during anaerobic Fe(III) oxide respiration are synthesized by an as yet unidentified biosynthetic pathway.

Previous studies demonstrated that purified complexes of MtrC and OmcA displayed soluble organic-Fe(III) reductase activity (Shi et al., 2006). *S. oneidensis* mutants containing deletions in genes encoding MtrC and OmcA (or both), however, retain the ability to reduce soluble organic-Fe(III) supplied as Fe(III)-NTA or Fe(III)-citrate (T. DiChristina, unpublished data). In the present study, ΔSO3034 also retained the ability to respire Fe(III)-citrate and Fe(III)-NTA at wild-type rates, an indication that the Fe(III)-hydroxamate reductase was not essential for Fe(III)-citrate or Fe(III)-NTA respiration, and was most likely dedicated to Fe(III) assimilation. The inability of wild-type *S. oneidensis* to respire Fe(III) chelated by the trihydroxamate ligand DEFB (log K=31 (Goodwin and Whitten, 1965)) as anaerobic electron acceptor corroborated this finding. *S. putrefaciens* strain 200 had previously displayed a similar pattern of soluble organic-Fe(III) respiratory capability (Haas and DiChristina, 2002). *S. putrefaciens* strain 200 reduced Fe(III)-citrate and Fe(III)-NTA (log K =11 and 16, respectively) but was unable to reduce soluble organic-Fe(III) substrates with log K>18 (Haas and DiChristina, 2002). This pattern of soluble organic-Fe(III) reduction activity may indicate that metal-reducing *Shewanella* only respire soluble organic-Fe(III) complexes with log K values less than a threshold level of approximately 18. By following this strategy, metal-reducing *Shewanella* may differentiate soluble organic Fe(III) complexes respired as anaerobic electron acceptor from those assimilated for nutrient purposes. Current work is

focused on application of complementary genetic, biochemical, and electrochemical techniques to identify the Fe(III)-chelating ligands produced by *S. oneidensis* during anaerobic Fe(III) oxide respiration, and to identify the terminal Fe(III) reductases that transfer electrons to the resulting soluble organic-Fe(III) complexes.

3.6 References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Arnow, L.E. (1937) Colorimetric determination of the components of 3,4-dihydroxyphenylalanine tyrosine mixtures. *Journal of Biological Chemistry* **118**: 531-537.

Bradford, M. (1976) Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Brendel, P.J., and Luther, G.W. (1995) Development of a gold amalgam volammetric microelectrode for the determination of dissolved Fe, Mn, O2 and S(-2) in porewaters of marine and freshwater sediments. *Environmental Science & Technology* **29**: 751-761.

Bristow, G., and Taillefert, M. (2008) VOLTINT: A Matlabg (R)-based program for semi-automated processing of geochemical data acquired by voltammetry. *Computers & Geosciences* **34**: 153-162.

Burnes, B.S., Mulberry, M.J., and DiChristina, T.J. (1998) Design and application of two rapid screening techniques for isolation of Mn(IV) reduction-deficient mutants of *Shewanella putrefaciens*. *Applied and Environmental Microbiology* **64**: 2716-2720.

Burns, J.L., and DiChristina, T.J. (2009) Anaerobic respiration of elemental sulfur and thiosulfate by *Shewanella oneidensis* MR-1 requires psrA, a homolog of the phsA gene of *Salmonella enterica* Serovar Typhimurium LT2. *Applied and Environmental Microbiology* **75**: 5209-5217.

Caldwell, C.D., and Crumbliss, A.L. (1998) Molecular recognition of ferrioxamine B by host-guest complex formation with lasalocid A in chloroform. *Inorganic Chemistry* **37**: 1906-1912.

Carey, E.A., and Taillefert, M. (2005) The role of soluble Fe(III) in the cycling of iron and sulfur in coastal marine sediments. *Limnology and Oceanography* **50**: 1129-1141.

Coates, J.D., Cole, K.A., Chakraborty, R., O'Connor, S.M., and Achenbach, L.A. (2002) Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. *Applied and Environmental Microbiology* **68**: 2445-2452.

Crosa, J.H. (1989) Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiological Reviews* **53**: 517-530.

Crosa, J.H., and Walsh, C.T. (2002) Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiology and Molecular Biology Reviews* **66**: 223-249.

Dale, J.R., Wade, R., Jr., and Dichristina, T.J. (2007) A conserved histidine in cytochrome *c* maturation permease CcmB of *Shewanella putrefaciens* is required for anaerobic growth below a threshold standard redox potential. *Journal of Bacteriology* **189**: 1036-1043.

Dehio, C., and Meyer, M. (1997) Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. *Journal of Bacteriology* **179**: 538-540.

DiChristina, T.J., and DeLong, E.F. (1994) Isolation of anaerobic respiratory mutants of *Shewannella putrefaciens* and genetic analysis of mutants deficient in anaerobic growth on Fe3+. *Journal of Bacteriology* **176**: 1468-1474.

DiChristina, T.J., Moore, C.M., and Haller, C.A. (2002) Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires *ferE*, a homolog of the *pulE* (*gspE*) type II protein secretion gene. *Journal of Bacteriology* **184**: 142-151.

DiChristina, T.J., Fredrickson, J.K., and Zachara, J.M. (2005) Enzymology of electron transport: energy generation with geochemical consequences. *Reviews in Mineralogy and Geochemistry* **59**: 27-52.

Duckworth, O.W., and Martin, S.T. (2001) Surface complexation and dissolution of hematite by C-1-C-6 dicarboxylic acids at pH=5.0. *Geochimica Et Cosmochimica Acta* **65**: 4289-4301.

Finn, R.D., Tate, J., Mistry, J., Coggill, P.C., Sammut, S.J., Hotz, H.R. et al. (2008) The Pfam protein families database. *Nucleic Acids Research* **36**: D281-D288.

Goodwin, J.F., and Whitten, C.F. (1965) Chelation of ferrous sulphate solutions by desferrioxamine B. *Nature* **205**: 281-283.

Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A. et al. (2006) Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 11358-11363.

Haas, J.R., and DiChristina, T.J. (2002) Effects of Fe(III) chemical speciation on dissimilatory Fe(III) reduction by *Shewanella putrefaciens*. *Environmental Science and Technology* **63**: 373-380.

Hau, H.H., and Gralnick, J.A. (2007) Ecology and biotechnology of the genus *Shewanella*. *Annual Review of Microbiology* **61**: 237-258.

Hernandez, M.E., Kappler, A., and Newman, D.K. (2004) Phenazines and other redox-active antibiotics promote microbial mineral reduction. *Applied and Environmental Microbiology* **70**: 921-928.

Hersman, L., Lloyd, T., and Sposito, G. (1995) Siderophore-promoted dissolution of hematite. *Geochimica Et Cosmochimica Acta* **59**: 3327-3330.

Hjerde, E., Lorentzen, M., Holden, M., Seeger, K., Paulsen, S., Bason, N. et al. (2008) The genome sequence of the fish pathogen *Aliivibrio salmonicida* strain LFI1238 shows extensive evidence of gene decay. *BMC Genomics* **9**: 616.

Holmen, B.A., and Casey, W.H. (1996) Hydroxamate ligands, surface chemistry, and the mechanism of ligand-promoted dissolution of goethite [alpha-FeOOH(s)]. *Geochimica Et Cosmochimica Acta* **60**: 4403-4416.

Hulo, N., Bairoch, A., Bulliard, V., Cerutti, L., De Castro, E., Langendijk-Genevaux, P.S. et al. (2006) The PROSITE database. *Nucleic Acids Research* **34**: D227-D230.

Kalinowski, B.E., Liermann, L.J., Givens, S., and Brantley, S.L. (2000) Rates of bacteria-promoted solubilization of Fe from minerals: a review of problems and approaches. *Chemical Geology* **169**: 357-370.

Kerisit, S., Rosso, K.M., Dupuis, M., and Valiev, M. (2007) Molecular computational investigation of electron-transfer kinetics across cytochrome-iron oxide interfaces. *Journal of Physical Chemistry C* **111**: 11363-11375.

Kostka, J.E., Luther III, G.W., and Nealson, K.H. (1995) Chemical and biological reduction of Mn(III)-pyrophosphate complexes: Potential importance of dissolved Mn(III) as an environmental oxidant. *Geochimica Et Cosmochimica Acta* **59**: 885-894.

Ledyard, K.M., and Butler, A. (1997) Structure of putrebactin, a new dihydroxamate siderophore produced by *Shewanella putrefaciens*. *Journal of Biological Inorganic Chemistry* **2**: 93-97.

Logan, B.E., Hamelers, B., Rozendal, R., Schrorder, U., Keller, J., Freguia, S. et al. (2006) Microbial fuel cells: methodology and technology. *Environmental Science & Technology* **40**: 5181-5192.

Lovley, D.R., and Phillips, E.J.P. (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology* **51**: 683-689.

Lovley, D.R., and Woodward, J. (1996) Mechanisms for chelator stimulation of microbial Fe(III)-oxide reduction. *Chemical Geology* **132**: 19-24.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. *Advances in Microbial Physiology* **49**: 219-285.

Madigan, M., Martinko, J., and Parker, J. (2003) *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Prentice Hall/Pearson Education.

Marshall, M.J., Plymale, A.E., Kennedy, D.W., Shi, L., Wang, Z., Reed, S.B. et al. (2008) Hydrogenase- and outer membrane *c*-type cytochrome-facilitated reduction of technetium(VII) by *Shewanella oneidensis* MR-1. *Environ Microbiol* **10**: 125-136.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., and Bond, D.R. (2008) *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 3968-3973.

Matzanke, B.F., Anemuller, S., Schunemann, V., Trautwein, A.X., and Hantke, K. (2004) FhuF, part of a siderophore-reductase system. *Biochemistry* **43**: 1386-1392.

Moeck, G.S., and Coulton, J.W. (1998) TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Molecular Microbiology* **28**: 675-681.

Montgomery, H., and Dymock, J.F. (1962) The rapid determination of nitrite in fresh and saline waters. *Analyst*: 374-378.

Myers, C.R., and Nealson, K.H. (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* **240**: 1319-1321.

Myers, C.R., and Myers, J.M. (1992) Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *Journal of Bacteriology* **174**: 3429-3438.

Neilands, J.B. (1995) Siderophores - structure and function of microbial iron transport compounds. *Journal of Biological Chemistry* **270**: 26723-26726.

Newman, D.K., and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. *Nature* **405**: 94-97.

Payne, A.N., and DiChristina, T.J. (2006) A rapid mutant screening technique for detection of technetium [Tc(VII)] reduction-deficient mutants of *Shewanella oneidensis* MR-1. *Fems Microbiology Letters* **259**: 282-287.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435**: 1098-1101.

Renshaw, J., Robson, G., Trinci, A., Wiebe, M., Livens, F., Collison, D., and Taylor, R. (2002) Fungal siderophores: structures, fuctions and applications. *Mycological Research* **106**: 1123-1142.

Roden, E.E. (2006) Geochemical and microbiological controls on dissimilatory iron reduction. *Comptes Rendus Geoscience* **338**: 456-467.

Ross, D.E., Brantley, S.L., and Tien, M. (2009) Kinetic characterization of terminal reductases OmcA and MtrC involved in respiratory electron transfer for dissimilatory iron reduction in *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* **75**: 5218-5226

Ross, D.E., Ruebush, S.S., Brantley, S.L., Hartshorne, R.S., Clarke, T.A., Richardson, D.J., and Tien, M. (2007) Characterization of protein-protein interactions involved in iron reduction by *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* **73**: 5797-5808.

Royer, R.A., Dempsey, B.A., Jeon, B.H., and Burgos, W.D. (2004) Inhibition of biological reductive dissolution of hematite by ferrous iron. *Environmental Science & Technology* **38**: 187-193.

Ruebush, S.S., Icopini, G.A., Brantley, S.L., and Tien, M. (2006) In vitro enzymatic reduction kinetics of mineral oxides by membrane fractions from *Shewanella oneidensis* MR-1. *Geochimica Et Cosmochimica Acta* **70**: 56-70.

Schroder, I., Johnson, E., and de Vries, S. (2003) Microbial ferric iron reductases. *FEMS Microbiology Reviews* **27**: 427-447.

Schwyn, B., and Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47-56.

Shi, L., Squier, T.C., Zachara, J.M., and Fredrickson, J.K. (2007) Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter:* a key role for multihaem *c*-type cytochromes. *Molecular Microbiology* **65**: 12-20.

Shi, L., Chen, B.W., Wang, Z.M., Elias, D.A., Mayer, M.U., Gorby, Y.A. et al. (2006) Isolation of a high-affinity functional protein complex between OmcA and MtrC: Two outer membrane decaheme *c*-type cytochromes of *Shewanella oneidensis* MR-1. *Journal of Bacteriology* **188**: 4705-4714.

Stookey, L.L. (1970) Ferrozine - A new spectrophotometic reagent for iron. *Analytical Chemistry* **42**: 779-&.

Taillefert, M., Luther III, G.W., and Nuzzio, D.B. (2000) The application of electrochemical tools for in-situ measurements in aquatic systems: a review. *Electroanalysis* **12**: 401-412.

Taillefert, M., Beckler, J.S., Carey, E.A., Burns, J.L., Fennessey, C.M., and DiChristina, T.J. (2007) *Shewanella putrefaciens* produces an Fe(III)-solubilizing ligand during anaerobic respiration on insoluble Fe(III) oxides *Journal of Inorganic Biochemistry* **101**: 1760-1767.

Tercier-Waeber, M.-L., and Taillefert, M. (2008) Remote in situ voltammetric techniques to characterize the biogeochemical cycling of trace metals in aquatic systems. *Journal of Environmental Monitoring* **10**: 30-54.

Vraspir, J.M., and Butler, A. (2009) Chemistry of marine ligands and siderophores. *Annual Review of Marine Science* **1**: 43-63.

Wade, R., and DiChristina, T.J. (2000) Isolation of U(VI) reduction-deficient mutants of *Shewanella putrefaciens*. *FEMS Microbiology Letters* **184**: 143-148.

Winkelmann, G., Schmid, D.G., Nicholson, G., Jung, G., and Colquhoun, D.J. (2002) Bisucaberin - A dihydroxamate siderophore isolated from *Vibrio salmonicida*, an important pathogen of farmed Atlantic salmon (*Salmo salar*). *BioMetals* **15**: 153-160.

Wirgau, J.I., Spasojevic, I., Boukhalfa, H., Batinic-Haberle, I., and Crumbliss, A.L. (2002) Thermodynamics, kinetics, and mechanism of the stepwise dissociation and formation of tris(L-lysinehydroxamato)iron(III) in aqueous acid. *Inorganic Chemistry* **41**: 1464-1473.

Xiong, Y.J., Shi, L., Chen, B.W., Mayer, M.U., Lower, B.H., Londer, Y. et al. (2006) High-affinity binding and direct electron transfer to solid metals by the *Shewanella oneidensis* MR-1 outer membrane *c*-type cytochrome OmcA. *Journal of the American Chemical Society* **128**: 13978-13979.

Yang, Y., Harris, D., Luo, F., Wu, L., Parsons, A., Palumbo, A., and Zhou, J. (2008) Characterization of the *Shewanella oneidensis* Fur gene: roles in iron and acid tolerance response. *BMC Genomics* **9**: S11.

Zinder, B., Furrer, G., and Stumm, W. (1986) Coordination chemistry of weathering kinentics of the surface .2. Dissolution of Fe(III) oxides. *Geochimica Et Cosmochimica Acta* **50**: 1861-1869.

CHAPTER 4

TYPE II PROTEIN SECRETION IS REQUIRED FOR FE(III) SOLUBILIZATION DURING ANAEROBIC FE(III) RESPIRATION BY *SHEWANELLA ONEIDENSIS* MR-1

4.1 Abstract

Microbial iron respiration is an integral process in the geochemical cycling of metals yet the molecular mechanism of this process remains poorly understood. Previous studies indicate that metal reducing bacteria such as *Shewanella oneidensis* MR-1 employ a strategy in which iron is solubilized by bacterially synthesized chelator molecules during anaerobic Fe(III) respiration. Random point mutants deficient in Fe(III)-solubilization and respiration (designated d29 and d64) were generated to determine which genes were involved in the solubilization process. Identification of the mutated genes reveal mutations in *gspE* and *gspG*, indicating that the type II secretion system is required not only for Fe(III) respiration (as determined in previous studies) but also for Fe(III) solubilization. Soluble Fe(III) is detected in the supernatant of WT cells, but not in the supernatant of d29 and d64 cells grown under identical conditions. These results further confirm that type II secretion plays a vital role in iron solubilization and respiration.

4.2 Introduction

Microbial electron transfer during metal respiration is a critical component to a variety of environmental and energy-generating processes such as geochemical cycling of metals, weathering of clays and minerals, plays a key role in microbial fuel cells and is intrinsically involved in bioremediation strategies for the reductive precipitation of radionuclides (such as uranium and transuranics) in contaminated environments (Lovley et al., 2004; DiChristina et al., 2005; Logan et al., 2006) Additionally, microbial metal respiration is hypothesized to be one of the earliest respiratory processes to have evolved on early earth (Pace, 1991; Holm, 1992). Therefore, determining the molecular mechanism of this terminal electron transfer is key to understanding at a fundamental level the processes in which metal reduction is involved. For sparingly soluble metals such as Fe(III), the mechanism by which this electron transfer occurs is poorly understood, as Fe(III) is most commonly found as a solid at circumneutral pH, and therefore cannot come into direct contact with the microbial inner membrane (the site of reduction for soluble terminal electron acceptors in gram negative bacteria) (Madigan et al., 2003; Lovley et al., 2004). Metal reducing bacteria must therefore utilize an alternate strategy for respiration on solid metal oxides. Postulated respiratory mechanisms for respiration on solid metals include: 1. localization of the metal reductase machinery onto the outer membrane such that it comes into direct contact with the extracellular metal ((Myers and Myers, 1992; DiChristina et al., 2002; Reguera et al., 2005; Gorby et al., 2006; Shi et al., 2006; Xiong et al., 2006; Wang et al., 2008; Meitl et al., 2009); 2. use of exogenous or endogenously synthesized electron shuttles which transfer electrons from the terminal metal reductase to the extracellular metal ((Newman and Kolter, 2000;

Hernandez and Newman, 2001; Coates et al., 2002; Hernandez et al., 2004; Marsili et al., 2008); or 3. cellular synthesis and secretion of metal chelating molecules into the extracellular milieu where they bind to metal atoms and are subsequently taken up by the cell and reduced at a metal reductase (Payne and DiChristina, 2006; Taillefert et al., 2007).

Previous studies have demonstrated production of an organic ligand by *Shewanella oneidensis* MR-1 (a metal-reducing gram-negative λ-proteobacteria) under anaerobic, Fe(III)-respiratory conditions which solubilizes solid Fe(III) (Taillefert et al., 2007; Jones et al., 2010). Initially, it was speculated that this ligand was a siderophore: a small Fe(III)-chelating molecule synthesized by a variety of fungi and bacteria for Fe(III) assimilation. In-frame gene deletion analysis of the siderophore biosynthetic and reductase genes indicated that the soluble Fe(III) observed in anaerobic incubations of *S.oneidensis* with solid iron is not a siderophore, but rather a novel molecule not associated with the siderophore biosynthetic system (Fennessey et al., 2010). Chemical mutagenesis was applied to identify the genes involved in the production of the anaerobic solubilizing molecule. Mutants were generated from wild-type *S.oneidensis*, each of which contained a single nucleotide change, and were screened using a high-throughput microelectrode-screening assay (MESA) (Jones et al., 2010). Using this method, two mutants were identified using this method which are unable to either produce soluble Fe(III) or reduce it when incubated anaerobically with HFO as terminal electron acceptor. These mutants were designated d29 and d64. The main objective of this study was to identify genes involved in Fe(III) solubilization during Fe(III) reduction.

4.3 Materials and Methods

Growth media and cultivation conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. For genetic manipulations, *S.oneidensis* strains were cultured at 30°C in Luria-Bertani (LB) medium (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone). For anaerobic growth experiments, *S. oneidensis* cultures were grown in a defined salts medium (SM) (DiChristina and DeLong, 1994) supplemented with lactate (18 mM) or formate (30 mM) as carbon/energy source under a nitrogen atmosphere. When required, chloramphenicol was supplemented to a final concentration of 25 μg mL^{-1}. *Escherichia coli* β2155 λ pir (Dehio and Meyer, 1997) was grown on LB medium supplemented with diaminopimelate (DAP) at a final concentration of 100 μg mL^{-1}.

Table 4.1. Strains and plasmids used in this study.

Strain	Features	Source
Shewanella oneidensis		
MR-1	Wild-type strain	ATCC
ΔgspD	in-frame deletion mutant	(Burns and DiChristina, 2009)
d29	random chemical mutant	(Jones et al., 2010)
d64	random chemical mutant	(Jones et al., 2010)
D6	d29 complementation mutant	This study
D6-4	d29 complementation mutant	This study
B8	d64 complementation mutant	This study
B8-3	d64 complementation mutant	This study
Escherichia coli		
β 2155 λ pir	*thrB1*004*pro thi strA hsdS lacZ*Δ*M15* (F9 *lacZ*Δ*M15 lacI*q *traD36 proA*1 *proB*1 Δ*dapA::erm pir::*RP4 Km^R	(Dehio and Meyer, 1997)
Plasmids		
pBBR1MCS	4.7 kb γR6K, *mob*RP4 Cm^R *lacZ*	(Kovach et al., 1994)
pVK100	20 kb Tet^R Kan^R Tra^+	(Knauf and Nester, 1982)

Determination of respiratory capability.

S. oneidensis WT, d29 and d64 mutant and transconjugate strains were inoculated in SM liquid growth medium (DiChristina and DeLong, 1994) (initial concentration of 10^7 cells mL^{-1}) amended with 18 mM lactate and10 mM fumarate, 50 mM Fe(III)-citrate, or 40 mM hydrous Fe(III)-oxide (HFO) as electron acceptor. Stock solutions of each electron acceptor were prepared according to previously described procedures (Saffarini et al., 1994; Blakeney et al., 2000; Taratus et al., 2000; Payne and DiChristina, 2006; Neal et al., 2007). Growth on fumarate was monitored via absorbance at 600 nm. Fe(III) reduction rates were determined by monitoring total Fe(II) production over time via HCl extraction followed by the Ferrozine colorimetric assay (Stookey, 1970). Cell growth was monitored by direct counts of acridine orange-stained cells via epifluorescence microscopy (Carl Zeiss AxioImager Z1 Microscope).

Clone bank construction.

Broad-host range cosmid pVK100 (Knauf and Nester, 1982) was isolated and purified from *E. coli* host strain EC100D via previously described CsCl density centrifugation (Garger et al., 1983). Genomic DNA was isolated from *S. oneidensis* WT cells grown aerobically in LB for 16 hr using the Wizard Genomic DNA Purification Kit (Promega). *S. oneidensis* WT genomic DNA was partially digested with Hin*d*III (New England Biolabs) for times ranging from 1-45 min. The digestion reactions were pooled, separated electrophoretically on a 0.9% LMP agarose gel, and the 10-20 kb DNA fragments were extracted from the gel, purified via phenol/chloroform extraction, and precipitated with EtOH and NaAc. pVK100 was fully digested with Hin*d*III (New England Biolabs) and dephosphorylated with antarctic phosphatase (New England

Biolabs). Digested/dephosphorylated pVK100 was purified via phenol/chloroform extraction and ligated to the partially digested S. oneidensis WT DNA fragments with T4 DNA ligase (NEB). The final ligation products were checked for long chain concatomers on a 1% agarose gel. Recombinant pVK100 was packaged into phage heads using the Max Plax lambda phage packaging kit (Epicentre). *E. coli* β 2155 λ *pir* cells were incubated with the packaged phage solution at 37°C for 20 min without shaking and subsequently diluted with LB amended with DAP and incubated at 37°C for an additional 1.5 hr. Serial dilutions were spread on LB agar supplemented with DAP and tetracycline, incubated at 37°C overnight, and the resulting colonies were pooled in LB and stored at -80°C.

Identification of the smallest complementing DNA fragment via genetic complementation analysis.

Mutants d29 and d64 were mated biparentally with the *S. oneidensis* gene clone bank harbored in *E.coli* β 2155 λ *pir*. Recipient and donor cells were grown to late log phase in appropriate growth medium, and harvested and washed twice in LB medium lacking antibiotics. Cells were mixed in a 5:1 donor: recipient ratio and spotted onto LB medium for 18-24 hr. The mating mix was serially diluted and transferred to LB agar supplemented with tetracycline (DAP was omitted to select against the *E. coli* β 2155 λ *pir* donor strain) and incubated at 30°C for 48 hr. The resulting d29 and d64 transconjugate colonies were subsequently tested for the restored ability to solubilize HFO via the MicroElectrode Screening Array (MESA) system, a high throughput screening system comprised of 8 100 μm diameter gold-mercury (Au/Hg) amalgam working electrodes and eight permanent 0.5 mm diameter Ag/AgCl reference and 0.5 mm

diameter platinum counter electrodes mounted on a manual micromanipulator (Jones et al., 2010). Recombinant pVK100 cosmids harbored in the d29 and d64 transconjugates with restored Fe(III)-solubilization activity were isolated (Sigma Aldrich plasmid isolation kit), and the complementing *S. oneidensis* WT DNA fragments were digested with Hin*d*III and ligated to cloning vector pBBR1MCS with T4 DNA ligase (NEB). The recombinant pBBR1MCS vectors were electroporated into *E. coli* β 2155 λ *pir* and transformants selected on LB agar supplemented with DAP, chloramphenicol and x-gal (for blue/white screening). The smallest complementing DNA fragments were identified via biparental mating and MESA-based screening of the resulting transconjugates for restored Fe(III) solubilization activity (biparental mating and MESA procedures were identical to those described above for genetic complementation analysis). Subclones displaying restored Fe(III) solubilization activity were isolated (Sigma-Aldrich plasmid isolation kit) and PCR-amplified using primers SeqF and SeqR (table 4.2). The ends of the PCR fragments were sequenced (Nevada Genomics Center, University of Nevada, Reno) and genes harbored on each subclone identified via BLAST analysis (Altschul et al., 1997) Individual genes harbored on each subclone were PCR-amplified using primers for *gspE*, *gspF*, *gspG* and *gspH* listed in Table 4.2, and ligated to pBBR1MCS. The pBBR1MCS constructs were electroporated into *E. coli* strain β 2155 λ *pir*, and the transformants were subsequently mated with mutants d29 and d64. The resulting d29 and d64 transconjugates were screened via MESA for restored Fe(III)-solubilization and reduction activities.

Table 4.2 Primers used in this study

Primer	Sequence
seqF	GAC TCG ACT CAC TAT AGG GCG AAT TGG A
seqR	GAC TGC TAT GAC CAT GAT TAC GCC AAG C
gspEF	GAC TGT CGA CCC TCT TCT AGC AAG AAG ACC AAG
gspER	GAC TGG ATC CTC AAA TGC TGG CAT GGC TTA CTC
gspFF	GAC TGT CGA CTC TGG AAG AAG TCT TGA GGG TGA
gspFR	GAC TGG ATC CTT ACT GAG AGG CAA CAT CCC A
gspGF	GAC TCT CGA GTA GGA GAT GTA GTC GAT GCA AAT G
gspGR	GAC TGT CGA CGG CGC AGC ATT AAC ATC TTA TTG
gspHF	GAC TGT CGA CTA AGA TGT TAA TGC TGC GCC ACG
gspHR	GAC TGG ATC CCA TTC CCC TAG CGT ATT TCA TCG G

Outer Membrane Protein Isolation.

Mutant and wild-type cells were grown in Westlake medium anaerobically with lactate (18 mM) as electron donor and fumarate (10 mM) as terminal electron acceptor to a late log phase (corresponding to a final cell density of 10^8 cells mL^{-1}). Proteins loosely attached to the outer membrane leaflet were removed from the cell surface via a KCl wash method (Ohlendieck, 1996). Briefly, cells in late log phase were centrifuged for 15 minutes at 10,000 x *g* (Sorvall RC5C) and resuspended in a KCl wash (0.5 M KCl, 100 mM Tris HCl, 1 mM EDTA; pH 7.0). The cell suspension was incubated under gentle stirring overnight followed by centrifugation at 10,000 x *g* (Sorvall RC5C). The resultant supernatant was passed through a syringe filter to remove any remaining whole cells.

Identification of Reduction and Solubilization Activity in Outer Membrane Wash and Supernatant.

Outer membrane proteins from wild-type, Δ*gspD*, d29, and d64 were screened for iron reduction activity. 50 μL of each outer membrane wash fraction was incubated anaerobically for 48 hr with 40 mM HFO in a 1X minimal salts buffer (pH 7.5) amended with 18 mM lactate in a 2% H_2 atmosphere. Iron reduction was monitored colorimetrically via the Ferrozine assay (Stookey, 1970). Identical incubations were prepared and Fe(III) solubilization activity was screened via the MESA system described previously.

WT MR-1, Δ*gspD*, d29 and d64 were grown anaerobically in a 2% H_2 atmosphere to late log phase on SM salts medium with 18 mM lactate and either 20 mM fumarate or 20 mM fumarate and 40 mM HFO as terminal electron acceptor. After 24 hours, each batch was centrifuged at 10,000 x *g* for 5 min, and filtered to remove any remaining whole cells and solid iron. Filtrates were loaded onto a 96-well plate screened immediately via the MESA system to identify the presence of soluble Fe(III) in solution.

4.4 Results

The clone bank was constructed and consists of the auxotrophic *E. coli* strain β 2155 λ *pir* containing the mobile vector pVK100 harboring 20kb fragments of partially digested wild-type DNA. Complete digestion reveals that each 20kb fragment is unique and represents different regions of the *S. oneidensis* MR-1 genome (data not shown).

The MESA system was used to identify two transconjugates with restored Fe(III)-solubilization activity: D6 (restoring activity to d29) and B8 (restoring activity to d64) which contained an approximately 20kb fragment of partially digested wild-type genome

which complemented each mutation. Subsequent subcloning revealed that both d29 and d64 were complemented by a 3.5kb fragment of wild-type genome, which was designated D6-4 and B8-3 respectively (Figure 4.1).

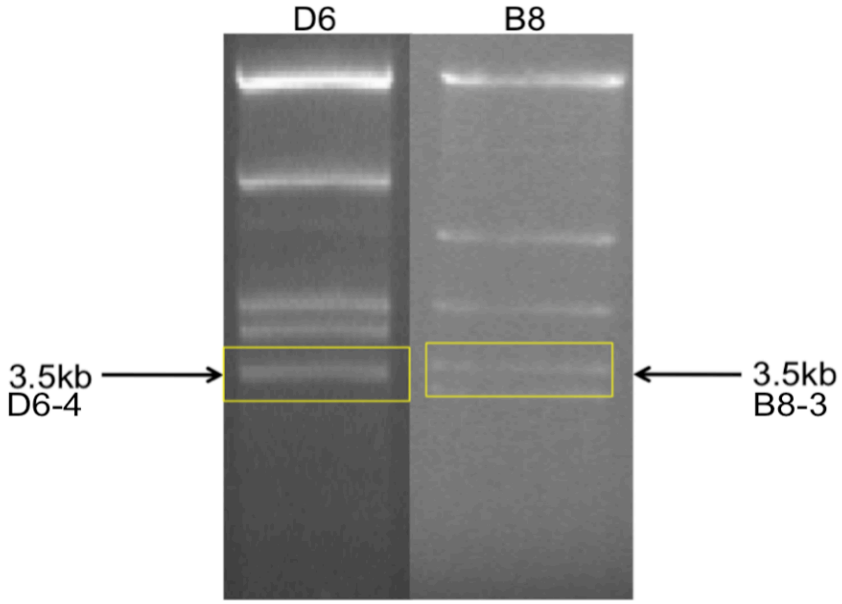

Figure 4.1 Agarose gel of Hin*d*III digest of the d29 complementing fragment D6 and the d64 complementing fragment B8. The smallest complementing fragments D6-4 and B8-3 are highlighted.

These fragments were sequenced and subsequently analyzed via BLAST (Altschul et al., 1997) and both 3.5kb fragments were identified as genes that correspond to the region *gspE – gspH*. Screening of d29 and d64 transconjugates containing each of the genes in the *gspE – gspH* region revealed that *gspE* (the ATPase required for type II secretion) restored activity to d64 and *gspG* (a component of the pseudopilus) restored d29 when screened via the MESA system. Transconjugates and mutants were grown anaerobically on HFO to determine Fe(III)-respiratory capability and all transconjugate strains (D6, D6-4, B8, B8-3, d29/*gspG* and d64/*gspE*) reduced HFO at wild-type rates

(Figure 4.2) and solubilized HFO at wild-type rates, as indicated by the peak at -4.5V (Figure 4.3), while the d29 and d64 mutants containing the other *gsp* genes demonstrated no reduction or solubilization activity (data not shown).

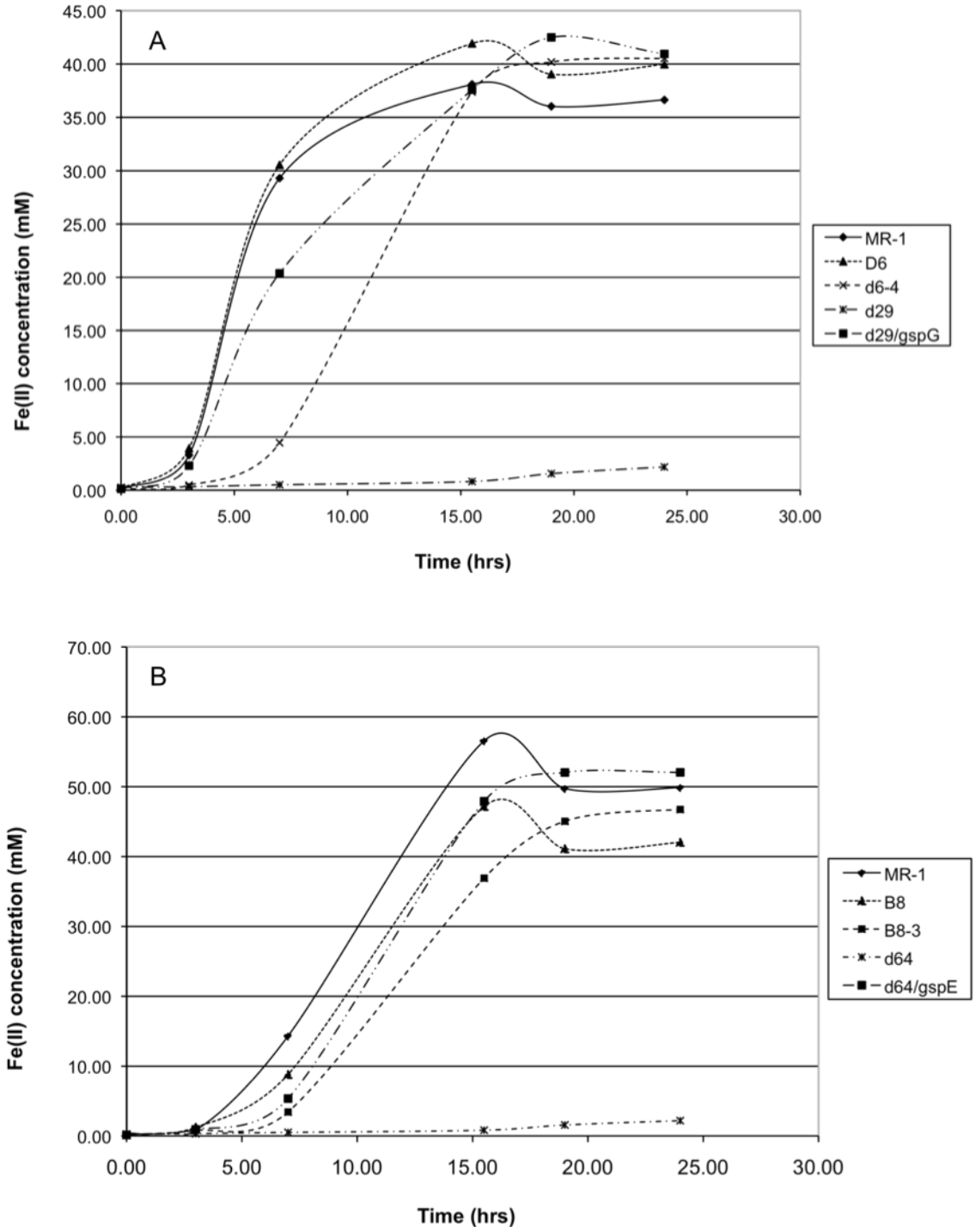

Figure 4.2 Anaerobic incubations of wild-type *S. oneidensis* (MR-1) and transconjugates with 40 mM HFO as terminal electron acceptor and 18 mM lactate as electron donor.

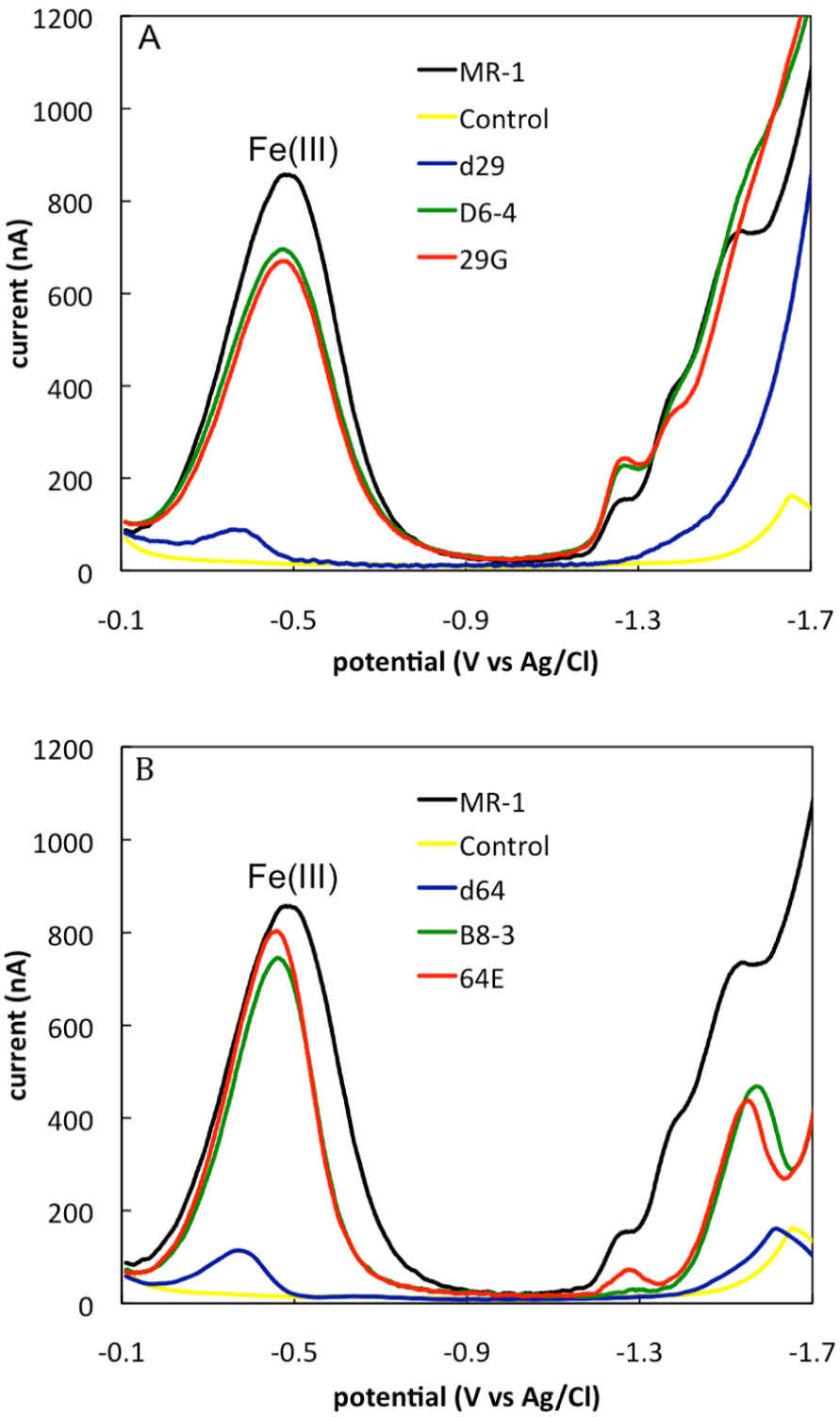

Figure 4.3 Soluble Fe(III) production of WT and mutants and transconjugates. A) WT, abiotic control, d29, D6-4, and d29/gspG. B) WT, abiotic control, d64, B8-3 and d64/gspE.

To determine whether soluble Fe(III) production could be detected in the outer membrane wash of WT cells but not of the type II secretion mutants, batches of cells were grown anaerobically and subjected to the KCl protein extraction procedure. Fe(III) reduction was observed in the outer-membrane wash of wild-type cells, but not in the wash of the type II mutants (Figure 4.4). Square-wave voltammetry screens revealed that soluble Fe(III) production did not occur in the outer membrane wash of either the wild-type or the mutant cells (data not shown).

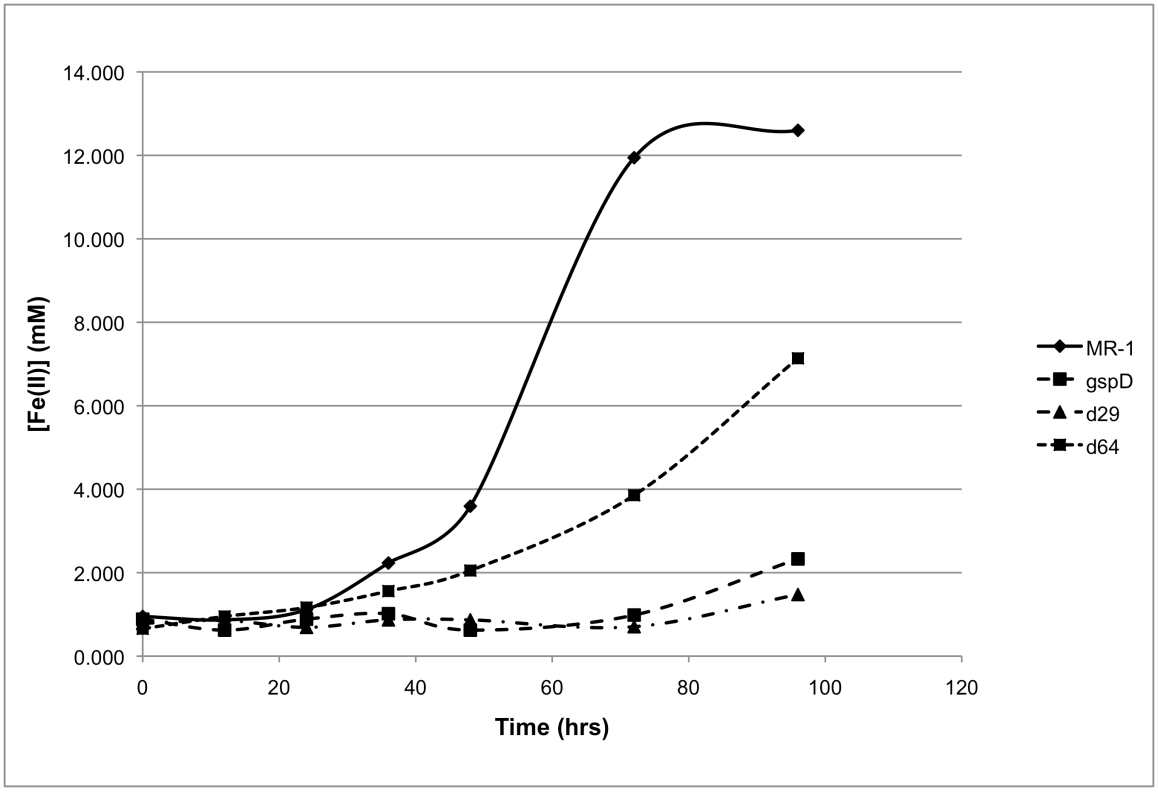

Figure 4.4 Anaerobic incubations of outer-membrane wash proteins with 40 mM HFO in the presence of 18mM lactate and H_2.

The spent supernatant of anaerobically grown WT, Δ*gspD*, d29, and d64 cells was screened via square wave voltammetry. Spent media of WT cells grown on HFO and fumarate displayed a strong soluble Fe(III) signal, while the supernatant of the type II

secretion mutants did not: WT MR-1 displayed a signal that was greater than 10x the signal produced by any mutant tested. Similarly, no soluble Fe(III) peaks were observed in the supernatant of the fumarate-only grown cells (Table 4.3). These results indicate that the soluble Fe(III) peak observed is not an artifact due to the presence of fumarate in the samples, and that the type (II) secretion system is necessary for the generation of soluble Fe(III). Square-wave voltammetry screens revealed that soluble Fe(III) production did not occur in the outer membrane wash of either the wild-type or the mutant cells.

Table 4.3 Soluble Fe(III) production detected via the MESA system in spent growth medium.

Organic-Fe(III) Production in cell supernatants		
Substrate	Strain	Organic-Fe(III) (nA)
Fumarate	MR-1	7.0 +/- 0.8
	gspD	7.4 +/- 1.0
	d29	9.7 +/- 0.6
	d64	8.4 +/- 0.0
2L-Ferrihydrite + Fumarate	MR-1	163.2 +/- 17.8
	gspD	12.3 +/- 11.6
	d29	4.3 +/- 0.4
	d64	3.8 +/- 0.2

4.5 Discussion

Type II secretion is a general protein secretion mechanism found in a broad range of microorganisms and is closely related to the type IV pilus secretion apparatus. It consists of a two-part secretion mechanism, first utilizing the *tat* or *sec* pathway to transport proteins across the inner membrane (Sandkvist, 2001). Once in the periplasmic space,

the signal peptide sequence is cleaved and the protein is folded. The second step in type II secretion involves a general secretory pathway mechanism to translocate proteins across the outer membrane. In *Shewanella*, the general secretory pathway (gsp) consists of 14 separate components: GspC, a structural domain which spans the periplasmic space, GspD, which makes up the porin in the outer membrane, GspE, the ATP-hydrolase which is responsible for driving protein secretion, GspLFMKNO, which form the in base of the secretory machinery embedded in the inner membrane and GspGHIJ, which form the pseudopilus apparatus which pushes the protein across the periplasmic space and outer membrane (Desvaux et al., 2004; Filloux, 2004; Economou et al., 2006; Dautin and Bernstein, 2007). The importance of type II secretion for metal respiration in *Shewanella* has been reported (DiChristina et al., 2002; Shi et al., 2008). The Gsp machinery in *S.oneidensis* MR-1 (or the homologous Pul machinery in *S.putrefaciens* strain 200) has been linked to the secretion of metal-reducing proteins onto the extracellular surface of the outer membrane. A 91kD iron-reducing protein complex was identified in *S.oneidensis* MR-1 which is absent on the outer membrane of Gsp (or Pul) mutants, further supporting the hypothesis that Fe(III)-reduction machinery is localized to the outer membrane using the type II secretion system (DiChristina et al., 2002). Later investigations demonstrated that the Fe(III)-reducing outer-membrane cytochromes MtrC and OmcA were localized on the outer membrane by the Gsp system (Shi et al., 2008)

Previous studies have also linked a solubilization mechanism to metal respiration in *S.oneidensis* MR-1 (Lovley and Woodward, 1996; Nevin and Lovley, 2002b, a). When wild-type cells are incubated with solid (in the form of HFO) or soluble iron (in the form of Fe(III)-citrate), electroactive soluble organic-Fe(III) complexes are detected by

square-wave voltammetry (Taillefert et al., 2007; Jones et al., 2010). Mutants incapable of solubilizing Fe(III) were generated (d29 and d64) and it was demonstrated that they were also incapable of metal reduction, despite retaining their ability to grow on all other terminal electron acceptors tested. This results indicates that the solubilization mechanism is an integral step in dissimilatory metal reduction.

In this study, the genes mutated in the Fe(III)-solubilization mutants d29 and d64 were identified to be type II secretion genes *gspG* and *gspE* respectively. Soluble Fe(III) was detected in the supernatant of wild-type cells grown anaerobically on fumarate and HFO but not in the presence of fumarate only nor in the supernatant of the type II secretion mutants, indicating that the type II system is required for Fe(III) solubilization. The fact that the type II secretion mechanism is required for Fe(III)-solubilization in two independently generated mutants suggests that it is a vital component of the solubilization process. Therefore, as it has been previously demonstrated that the type II secretion system is involved in the secretion of Fe(III)-reducing complexes, there is evidence that this same secretion system is also responsible for the Fe(III)-solubilization step in solid Fe(III) respiration. This offers significant insights into the molecular mechanism of Fe(III) solubilization prior to respiration as it indicates either that: 1. the type II secretion system transfers a component of the solubilization apparatus to the outer membrane, or 2. the type II secretion system is actively pumping the Fe(III) ligand out of the cell, representing the first report case of type II secretion involvement in small molecule trafficking.

4.6 References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Blakeney, M.D., Moulaei, T., and DiChristina, T.J. (2000) Fe(III) reduction activity and cytochrome content of *Shewanella putrefaciens* grown on ten compounds as sole terminal electron acceptor. *Microbiology Research* **155**: 87-94.

Burns, J.L., and DiChristina, T.J. (2009) Anaerobic respiration of elemental sulfur and thiosulfate by *Shewanella oneidensis* MR-1 requires psrA, a homolog of the phsA gene of *Salmonella enterica* Serovar Typhimurium LT2. *Applied and Environmental Microbiology* **75**: 5209-5217.

Coates, J.D., Cole, K.A., Chakraborty, R., O'Connor, S.M., and Achenbach, L.A. (2002) Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. *Applied and Environmental Microbiology* **68**: 2445-2452.

Dautin, N., and Bernstein, H.D. (2007) Protein secretion in gram-negative bacteria via the autotransporter pathway. *Annu Rev Microbiol* **61**: 89-112.

Dehio, C., and Meyer, M. (1997) Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. *Journal of Bacteriology* **179**: 538-540.

Desvaux, M., Parham, N.J., Scott-Tucker, A., and Henderson, I.R. (2004) The general secretory pathway: a general misnomer? *Trends in Microbiology* **12**: 306-309.

DiChristina, T.J., and DeLong, E.F. (1994) Isolation of anaerobic respiratory mutants of *Shewannella putrefaciens* and genetic analysis of mutants deficient in anaerobic growth on Fe3+. *Journal of Bacteriology* **176**: 1468-1474.

DiChristina, T.J., Moore, C.M., and Haller, C.A. (2002) Dissimilatory Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* requires *ferE*, a homolog of the *pulE* (*gspE*) type II protein secretion gene. *Journal of Bacteriology* **184**: 142-151.

DiChristina, T.J., Fredrickson, J.K., and Zachara, J.M. (2005) Enzymology of electron transport: energy generation with geochemical consequences. *Reviews in Mineralogy and Geochemistry* **59**: 27-52.

Economou, A., Christie, P.J., Fernandez, R.C., Palmer, T., Plano, G.V., and Pugsley, A.P. (2006) Secretion by numbers: Protein traffic in prokaryotes. *Molecular Microbiology* **62**: 308-319.

Fennessey, C.M., Jones, M.E., Taillefert, M., and DiChristina, T.J. (2010) Siderophores are not involved in Fe(III) solubilization during anaerobic Fe(III) respiration by *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* **76**: 2425-2432.

Filloux, A. (2004) The underlying mechanisms of type II protein secretion. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1694**: 163-179.

Garger, S.J., Griffith, O.M., and Grill, L.K. (1983) Rapid purification of plasmid DNA by a single centrifugation in a 2-step cesium-chloride ethidium-bromide gradient. *Biochemical and Biophysical Research Communications* **117**: 835-842.

Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A. et al. (2006) Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 11358-11363.

Hernandez, M.E., and Newman, D.K. (2001) Extracellular electron transfer. *Cellular and Molecular Life Sciences* **58**: 1562-1571.

Hernandez, M.E., Kappler, A., and Newman, D.K. (2004) Phenazines and other redox-active antibiotics promote microbial mineral reduction. *Applied and Environmental Microbiology* **70**: 921-928.

Holm, N.G. (1992) Why are hydrothermal systems proposed as plausible environments for the origin of life. *Origins of Life and Evolution of the Biosphere* **22**: 5-14.

Jones, M.E., Fennessey, C.M., DiChristina, T.J., and Taillefert, M. (2010) *Shewanella oneidensis* MR-1 mutants selected for their inability to produce soluble organic-Fe(III) complexes are unable to respire Fe(III) as anaerobic electron acceptor. *Environmental Microbiology* **12**: 938-950.

Knauf, V.C., and Nester, E.W. (1982) Wide host range cloning vectors - A cosmid clone bank of an *Agrobacterium* Ti plasmid. *Plasmid* **8**: 45-54.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., 2nd, and Peterson, K.M. (1994) pBBR1MCS: a broad-host-range cloning vector. *BioTechniques* **16**: 800-802.

Logan, B.E., Hamelers, B., Rozendal, R., Schrorder, U., Keller, J., Freguia, S. et al. (2006) Microbial fuel cells: methodology and technology. *Environmental Science & Technology* **40**: 5181-5192.

Lovley, D.R., and Woodward, J. (1996) Mechanisms for chelator stimulation of microbial Fe(III)-oxide reduction. *Chemical Geology* **132**: 19-24.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. *Advances in Microbial Physiology* **49**: 219-285.

Madigan, M., Martinko, J., and Parker, J. (2003) *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Prentice Hall/Pearson Education.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., and Bond, D.R. (2008) *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 3968-3973.

Meitl, L.A., Eggleston, C.M., Colberg, P.J.S., Khare, N., Reardon, C.L., and Shi, L. (2009) Electrochemical interaction of *Shewanella oneidensis* MR-1 and its outer membrane cytochromes OmcA and MtrC with hematite electrodes. *Geochimica Et Cosmochimica Acta* **73**: 5292-5307.

Myers, C.R., and Myers, J.M. (1992) Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *Journal of Bacteriology* **174**: 3429-3438.

Neal, A.L., Dublin, S.N., Taylor, J., Bates, D.J., Burns, L., Apkarian, R., and DiChristina, T.J. (2007) Terminal electron acceptors influence the quantity and chemical composition of capsular exopolymers produced by anaerobically growing *Shewanella* spp. *Biomacromolecules* **8**: 166-174.

Nevin, K.P., and Lovley, D.R. (2002a) Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. *Applied and Environmental Microbiology* **68**: 2294-2299.

Nevin, K.P., and Lovley, D.R. (2002b) Mechanisms for Fe(III) oxide reduction in sedimentary environments. *Geomicrobiology Journal* **19**: 141-159.

Newman, D.K., and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. *Nature* **405**: 94-97.

Ohlendieck, K. (1996) Extraction of membrane proteins. In *Protein Purification Protocols*. Doonan, S. (ed). Totowa, NJ: Humana Press, Inc., pp. 293-304.

Pace, N.R. (1991) Origin of life - Facing up to the physical setting. *Cell* **65**: 531-533.

Payne, A.N., and DiChristina, T.J. (2006) A rapid mutant screening technique for detection of technetium [Tc(VII)] reduction-deficient mutants of *Shewanella oneidensis* MR-1. *Fems Microbiology Letters* **259**: 282-287.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435**: 1098-1101.

Sandkvist, M., (2001) Biology of type II secretion. *Molecular Microbiology* **40**: 271-283.

Saffarini, D.A., DiChristina, T.J., and Nealson, K.H. (1994) Anaerobic respiration by *Shewanella putrefaciens* requires both chromosomal and plasmid-borne genes. *FEMS Microbiology Letters* **119**: 271-278.

Shi, L., Deng, S., Marshall, M.J., Wang, Z.M., Kennedy, D.W., Dohnalkova, A.C. et al. (2008) Direct involvement of type II secretion system in extracellular translocation of *Shewanella oneidensis* outer membrane cytochromes MtrC and OmcA. *Journal of Bacteriology* **190**: 5512-5516.

Shi, L., Chen, B.W., Wang, Z.M., Elias, D.A., Mayer, M.U., Gorby, Y.A. et al. (2006) Isolation of a high-affinity functional protein complex between OmcA and MtrC: Two outer membrane decaheme *c*-type cytochromes of *Shewanella oneidensis* MR-1. *Journal of Bacteriology* **188**: 4705-4714.

Stookey, L.L. (1970) Ferrozine - a new spectrophotometric reagent for iron. *Analytical Chemistry* **42**: 779-781.

Taillefert, M., Beckler, J.S., Carey, E.A., Burns, J.L., Fennessey, C.M., and DiChristina, T.J. (2007) *Shewanella putrefaciens* produces an Fe(III)-solubilizing ligand during anaerobic respiration on insoluble Fe(III) oxides *Journal of Inorganic Biochemistry* **101**: 1760-1767.

Taratus, E.M., Eubanks, S.G., and DiChristina, T.J. (2000) Design and application of a rapid screening technique for isolation of selenite reduction-deficient mutants of Shewanella putrefaciens. *Microbiology Research* **155**: 79-85.

Wang, Z.M., Liu, C.X., Wang, X.L., Marshall, M.J., Zachara, J.M., Rosso, K.M. et al. (2008) Kinetics of reduction of Fe(III) complexes by outer membrane cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology* **74**: 6746-6755.

Xiong, Y.J., Shi, L., Chen, B.W., Mayer, M.U., Lower, B.H., Londer, Y. et al. (2006) High-affinity binding and direct electron transfer to solid metals by the *Shewanella oneidensis* MR-1 outer membrane *c*-type cytochrome OmcA. *Journal of the American Chemical Society* **128**: 13978-13979.

CHAPTER 5

CONCLUSIONS

Conclusions

Shewanella oneidensis MR-1 is gram-negative proteobacterium capable of respiration of a broad range of terminal electron acceptors including radioactive and insoluble metals. Amongst these elements, Fe(III) has a solubility of $<10^{-25}$ at circumneutral pH, rendering it largely inaccessible to the gram-negative inner membrane: the site of the electron transport chain and respiratory apparatus. How *Shewanella* is capable of using this metal as a terminal electron acceptor for respiration therefore remains unclear. Previous investigations revealed that a cluster of outer membrane proteins are capable of Fe(III) reduction implying that *Shewanella* uses a direct contact mechanism in order to reduce Fe(III) under anaerobic respiratory conditions. Soluble Fe(III) has been detected in natural environments where *Shewanella* may play a key role in the microbial ecology of the system. Additionally, in vitro, soluble Fe(III) is detected when pure cultures of various strains of *Shewanella* are incubated with solid Fe(III), while abiotic controls with solid Fe(III) (in the form of HFO) and Fe(III)-citrate do not display soluble Fe(III) signals. Thus, under anaerobic Fe(III)-respiring conditions, *Shewanella* generates soluble Fe(III), presumably to facilitate respiration of a solid terminal electron acceptor.

Under Fe(III)-respiring conditions, evidence indicates that *Shewanella* produces an organic ligand molecule that chelates and solubilizes Fe(III) prior to respiration. This Fe(III)-ligand complex is detectable via square-wave voltammetry. Initial speculation on the identity of the ligand molecule prompted the consideration that the solubilizing ligand

observed during Fe(III) respiration is actually a siderophore. Deletion mutants were constructed in the siderophore biosynthesis gene SO3031 and in the siderophore reductase gene SO3034. Both mutants were capable of Fe(III) respiration and solubilization, indicating that the assimilatory Fe(III)-solubilization mechanism is distinct from the Fe(III)-solubilization machinery used during respiration. These results demonstrate that a separate soluble-Fe(III) reductase is used under Fe(III)-respiratory conditions than that used for Fe(III) assimilation. These data also indicate that two separate Fe(III)-solubilization mechanisms are employed by *Shewanella* depending on the growth conditions and the nutrient requirements of the cells.In order to determine the genes involved in the solubilization mechanism, wild-type *Shewanella oneidensis* MR-1 was subjected to random mutagenesis and a large-scale square-wave voltammetry screening system (MESA) was developed to detect production of soluble Fe(III). Two random mutants were identified using this large scale screening system which were incapable of producing soluble Fe(III) under Fe(III)-respiratory conditions: d29 and d64. Subsequent genetic complementation analysis revealed that both mutations occurred in the general secretion pathway (gsp), a type II secretion mechanism utilized by the cells to secrete proteins to the outer membrane or expel them into the extracellular space. This implies that the solubilization apparatus is secreted to the outer membrane during Fe(III)-respiration or that the Fe(III)-ligand is excreted by the gsp system. Loosely attached outer-membrane proteins were washed from the surface of the cells, and the wash was incubated with solid Fe(III) in the presence of Ferrozine and H_2. Reduction of the Fe(III) was observed in the outer membrane wash, but Fe(III)-solubilization was not. This may

indicate that a concerted mechanism between the membranes is required for Fe(III)-solubilization, or that whole cells are necessary for active Fe(III) solubilization activity.

How this model of microbial metal respiration fits with the current understanding of the system remains to be elucidated. One possible explanation is that microbial metal respiration occurs in phases, whereby there is an initial direct-contact electron transfer phase which triggers biosynthesis of Fe(III)-solublizing or electron shuttling complexes, allowing for respiration on a greater surface area of the iron-oxides and enabling cells which are not within direct electron transfer distance to also respire the metal. A second suggestion of how these two models could work in concert is that the identified surface-exposed Fe(III)-reducing cytochromes MtrC and OmcA may act as general Fe(III) reductants, transferring electrons both to solid iron oxides yet also acting as the terminal reductase of the organic ligand bound Fe(III). Overall, rather than contradicting the findings previously published on this subject, the work presented here instead reveals an aspect of the microbial metal respiratory system which heretofore was generally overlooked, and sheds further light on the workings of this complex machinery.

Future Directions

The work presented opens the door for exciting future directions for research in microbial metal respiration.

1. The bacterially-synthesized Fe(III)-solubilizing molecule should be identified. Despite the observation of Fe(III)-solubilization activity in various species of DMRBs, a microbially synthesized chelating molecule has yet to be identified. There may be various reasons for this: 1. the ligand is not excreted into the

extracellular milieu, but is in fact bound to the outer membrane of the cell via a fatty acid tail (similar to the structure of amphiphilic siderophores observed in some marine bacteria). 2. The ligand may be bound to the iron. Presumably, this ligand molecule has a high affinity for Fe(III), therefore when the supernatant (assumed to contain the solubilizing ligand) is separated from the cells and solid iron, the ligand is actually still bound to the iron and is not present in the supernatant. 3. The soluble Fe(III)-ligand complex is unstable and is therefore difficult to detect after extensive manipulations of the ligand-containing solutions.

2. The solubilizing proteins should be identified. The results of the fractionation studies appear to demonstrate that Fe(III)-solubilization is only carried out by whole cells; individual subcellular fractions were unable to solubilize Fe(III) when incubated with HFO. This suggested that a concerted mechanism amongst the membrane fractions is required: the solubilization mechanism is not simply localized to a single subcellular component.
3. The role of the ligand should be identified. It's presumed that *Shewanella* produces the Fe(III) solubilizing ligand in order to provide an easily accessible, high energy terminal electron acceptor to the cells. However, it is possible that the biosynthetic ligand is actually used to clear Fe(II) from the surface of the Fe(III)-oxides to provide better access to the Fe(III) atoms. Subsequent oxidation of the Fe(II)-ligand to Fe(III)-ligand (that which is detected in square-wave voltammetry screenings) may be a result of an abiotic reaction or an oxidation by the cells.

4. The role of the Fe(II) assimilatory system under anaerobic respiratory conditions should be studied. In the siderophore biosynthesis mutant SO3031, no growth deficiencies were observed even when the cells were grown under Fe(III)-starvation conditions. This suggests that the cells are accumulating iron (a required nutrient) via an alternate pathway from the standard siderophore Fe(III)-uptake system. *S.oneidensis* MR-1 contains Fe(II) uptake genes (the *Feo* gene cluster) and may use this system to accumulate iron for growth.

These are simply a few examples of the future directions research in this area may take. The work presented here represents just a brief initial glance into an exciting novel microbial respiratory system.